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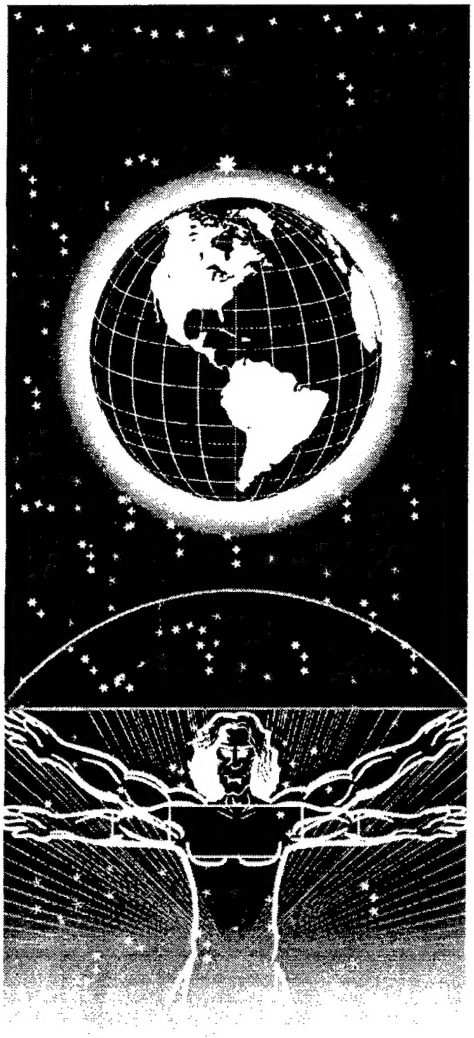

UNITED STATES AIR FORCE ARMSTRONG LABORATORY

A BACTERIAL TREATMENT SYSTEM FOR THE REMEDIATION OF TRICHLOROETHYLENE

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This report has been reviewed and is approved for publication.

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14. Abstract A genetically altered bacterium *Burkholderia (Pseudomonas) cepacia* PR123 and closely related genetic derivatives were tested for bioreactor and *in situ* trichloroethylene (TCE) degradation. PR123 was shown to degrade TCE in a plugged flow bioreactor, but failed to form a stable biofilm under test conditions at Hanscom AFB. Indigenous microorganisms dominated the reactors shortly after inoculation in every instance, despite changes in support matrix and primary carbon source. The continuous addition of the genetically altered bacterium did achieve a significant (>80%) removal of TCE and *cis*-dichloroethylene from the waste stream at concentrations of 500-800 *u*g/L, at 0.26 GPM, thus confirming the capacity the constitutively expressed toluene *ortho*-monooxygenase (Tom) to cooxidize TCE under environmental conditions. For this reason the Tom constitutive plasmid: TOM*31c* (a kanamycin resistant derivative of TOM), was transferred to two superior biofilm forming bacteria: *P. cepacia* 17616 and *P. sp* JS150, and dominant aquifer bacterium from Wichita KS WS23. These transconjugants also constitutively degraded TCE, but were no more competitive in biofilm reactors than PR123. In column tests >95% of the TCE was degraded in an 8 hour residence time.

15. Subject Terms Trichloroethylene, Bioremediation, Toluene, Phenol, Toluene Ortho Monooxygenase

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PREFACE

This report summarizes research conducted by the University of West Florida for Armstrong Laboratory through Firm Fixed Price Contract No F08635-92-C-0103, "A Bacterial Treatment System for the Remediation of Trichloroethylene", during the performance period 11/1/92 through 12/31/94.

The work summarized in this report has been conducted by Malcolm S. Shields. The project officer was Alison Thomas (Armstrong Labs). Jim Spain (Armstrong Labs) reviewed workplans and provided technical advice and scientific consultation throughout the project.

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EXECUTIVE SUMMARY

A. OBJECTIVE

In this project we have sought to test bioreactor and *in situ* applications of genetically altered versions of plasmid TOM derived from the bacterium *Burkholderia (Pseudomonas) cepacia* G4, which enable the constitutive cooxidation of trichloroethylene (TCE) by bacteria that maintain and express them.

The specific goals of this work were to test the effectiveness of this altered genetic element in two types of applications: above ground bioreactors, or above ground simulations of *in situ* treatments. These were carried out in three separate phases:

1. Phase I. Bioreactors. To test *B. cepacia* $PR1_{23}$ (TOM_{23c}) efficiency in a plugged-flow biofilm reactor using crushed oyster shell, diatomaceous earth (DE) pellets and ceramic pellets as colonizable substrate packing.

2. Phase II. Transconjugant Bacterial Vectors. To develop alternative bacterial hosts with greater biofilm colonization capabilities (*B. cepacia* 249 and *Pseudomonas* sp. JS150) or demonstrated aquifer dominance (WS23) that also constitutively express Tom while stably maintaining TOM_{31c}, and to test them under laboratory conditions. To test $PR1_{23}$ (TOM_{23c}), 17616 (TOM_{31c}) and JS150 (TOM_{31c}) as immobilized cells in a membrane-immobilized cell bioreactor and a fluidized bed reactor containing calcium-alginate bead-immobilized cells.

3. Phase III. *In Situ.* To determine the potential for TCE treatment *in situ* through introduction of $PR1_{23}$ (TOM_{23c}), 249 (TOM_{31c}) and JS150 (TOM_{31c}), and WS23 (TOM_{31c}) in simulated aquifer columns, under field conditions with native soil and untreated groundwater containing BTEX and TCE at NAS Whiting Field, FL.

B. BACKGROUND

G4, previously noted for its capacity to cooxidize TCE through the action of toluene *ortho*-monooxygenase (Tom) (the first component enzyme of the toluene/benzene/phenol degradative pathway of this organism), has been shown to maintain the Tom genes on a large self-transmissible plasmid designated TOM. The production of Tom in this strain occurs as a consequence of exposure to these chemicals, but not TCE. We have made several genetically altered forms of this plasmid which all constitutively express Tom and therefore constitutively cooxidize TCE, without the need for these toxic inducer chemicals. The bacterial plasmids used in this study, TOM $_{23c}$ and TOM$_{31c}$, are the result of introducing the kanamycin resistant transposon Tn5 into the native plasmid: TOM, and selecting for certain specific secondary insertions of the flanking IS50 elements. Since the literature surrounding Tn5 has come solely from studies in which *E. coli* was used as the host, EPA has ruled that bacteria carrying these plasmids represent "significantly altered organisms" and, as such cannot be intentionally released or used in the field without safeguards against their accidental release. We were therefore permitted to use these organisms in the field with the express requirement that they be contained in a "bioreactor" and that the effluent be treated with hypochlorite to prevent survival of the introduced bacteria. For this reason the "*in situ* study" had to be contained in "bioreactor" columns, but insofar as we were able, these simulated the oxygenated aquifer biological and chemical parameters.

These plasmids were developed in joint research projects including the US EPA Environmental Research Laboratory at Gulf Breeze, Florida; the Center for Environmental Diagnostics and Bioremediation, The University of West Florida, Pensacola Florida; and Technical Resources Inc. (on site research contractors for the US EPA in Gulf Breeze) over the last 8 years. We wished to determine if the genetic manipulations accomplished in the laboratory that resulted in the elimination of the need for toxic inducers to achieve TCE cooxidative metabolism in bacteria, could be developed into a workable field application system. Both bioreactor and *in situ* applications were explored. It was anticipated that since bioreactors are inherently more controllable than *in situ* systems, they would prove to be the most amenable to these genetic constructs. Therefore, bioreactors were investigated first and *in situ* applications second.

The latter months of this study overlapped with another Air Force project (funded through the Army Research Office, TCN 94109) in which we developed several potentially stable field application vectors for groundwater treatment *in situ*. We were able to take advantage of the scheduled *in situ* field test of the organisms developed under this agreement at NAS Whiting Field to include one of these new FAV's in this same study: WS23 (TOM$_{31c}$).

C. SCOPE

The period covered by this report includes research initiated in November 1992, and completed in November 1994. The work spanned two phases of research. Phase I was the development of these bacterial systems for use in fixed-film bioreactors. It was initiated with $PR1_{23}$ in which the reactor support matrix was crushed oyster shell. This was because initial lab results indicated the capacity of this bacterium to colonize this material to significant levels (i.e. $> 10^8$ cells per gram of material). Information obtained through tests with this organism in bench-scale bioreactors (20 L) was used to design a field test of the immobilized cells at Hanscom AFB, where TCE contaminated groundwater was already undergoing air-stripping treatment, in 100 L stainless steel bioreactors. Use of an Air Force mobile lab allowed the testing of two such field-scale reactors, packed and tested successively with crushed oyster shell, DE and ceramic pellets (type Z material from Grace Sierra Corporation) and inoculated with $PR1_{23}$.

Latter stages of phase I included the development of alternate hosts for $PR1_{31}$ that were previously recognized for their capacity to develop good biofilms, and that could also express Tom constitutively. These TCE degradative strains were tested for their ability to colonize DE in the lab in bench and development scale (1 L) bioreactors and for their ability to stably compete with indigenous bacteria present in TCE contaminated groundwater. Also tested was the capacity for PR123 to function in two other types of immobilized cell reactors. One was a spiral, membrane reactor designed to arrest cells and thus prevent their loss through dilution, and another was a fluidized bed reactor in which the active bacteria were first immobilized in beads of calcium alginate.

Phase II involved field testing five bacterial hosts harboring TOM_{31c} or TOM_{23c} in either 20 L cores containing native soil and receiving contaminated groundwater from a BTEX and TCE contaminated plume on NAS Whiting Field. The specific object of this test not being to optimize TCE degradation by these bacteria but rather to determine the survival of degradative activity of a specified number of inoculant bacteria under three regimes consisting of C, N & P; N & P; or no additions to oxygenated site groundwater.

D. METHODOLOGY

Column reactors were used to test plugged flow reactor applications to utilize a biofilm and continuous addition of cells. These were constructed in three sizes: 100 l stainless steel and 20 l and 3 l Glass reactors. The latter were also used as fluidized-bed reactors for encapsulated cells and as soil columns for the single passage of nutrient-amended contaminated site water. A spiral membrane reactor was also used in an attempt to imbed and immobilize cells introduced in a single pass flow-through design. The two larger models and the spiral reactor were configured to allow in-line monitoring of oxygen concentration, and recirculation flow rates.

E. TEST DESCRIPTION

The first phase involved the use of two 100 l stainless steel plugged-flow reactors (equipped with progressing cavity pumps for the rapid recirculation of column liquids) for the degradation of TCE in air and water, ostensibly by a biofilm of $PR1_{23}$ at Hanscom AFB. Failure to establish a biofilm led to the use of these recirculating reactors through the continuous addition of $PR1_{23}$, grown in batch and fed daily to the liquid treatment phase. Liquid samples removed before and after treatment were analyzed via purge and trap GC, gas samples by direct on column injection.

The second phase included the testing of two immobilization matrices in an attempt to prevent dilution loss of $PR1_{23}$ from a CSTR fluidized bed reactor. Oxygenation was by means of pure O_2 exposure to influent water in the laboratory prior to addition of TCE to the stream. Liquid samples removed before and after treatment were analyzed via purge and trap GC.

The third phase involved the modeling of an *in situ* recirculation application of the several strains in above ground soil columns at NAS Whiting Field. The goal here was to establish bacteria as they would be delivered to the aquifer in an *in situ* recirculation well, and measure the degradation of TCE under similar flow conditions in above ground columns. Liquid samples removed before and after treatment were analyzed via purge and trap GC.

F. RESULTS

1. Phase I: Bioreactor Applications

Despite capacity of $PR1_{23}$ to form an active biofilm, such was not achieved under field conditions at Hanscom AFB, Boston. The capacity for indigenous microorganisms to competitively dominate the reactors was seen in every instance, despite changes in support matrix and primary carbon source. The continuous addition of $PR1_{23}$ did result in a significant (>80%) removal of TCE and *cis*-dichloroethylene from the waste stream at concentrations of 500-800 μg/L, at 0.26 GPM. This proved the capacity of $PR1_{23}$ to cooxidize these substrates under environmental conditions. This strains inability to remain stable in the environment of the bioreactor seriously limits the application of this particular organism to a traditional fixed-film bioreactor. This necessitated a change in approach.

Since the bioreactors proved to have a very demanding ecological selection of their own, there seemed to be relatively little reason to pursue them further for two reasons. (1) It became apparent that we would not easily be able to control the bioreactor conditions to the point we could select for our particular degraders, since the mechanism we desired was cometabolic in nature. That is to say, the selection criteria imposed on the bioreactor were imposed by the primary carbon feed source (i.e. the necessary carbon and energy source) which resulted in rapid colonization of the bioreactor by the most competitive species present that could utilize this substrate. This seemed never to be one of the constructed strains. (2) *In situ* treatment systems are far more desirable than bioreactors due to difficulty of maintenance and cost of operation.

2. Phase II: *In Situ* Applications

For these reasons, efforts were centered on the *in situ* application, not of the strain PR1 ($_{23}$ or $_{31}$) but on the Tom constitutive plasmid: TOM_{31c} (a kanamycin resistant derivative of TOM genetically similar to TOM_{23c}). TOM_{31c} was transferred via conjugation to several alternative bacterial hosts: 249 and JS150 because of their published capacities to form active biofilms (capacities that were maintained by their TOM_{31c} bearing derivative). These newly constructed bacteria also constitutively degraded TCE. However, laboratory modeling of fixed film and immobilized cell bioreactors indicated that these organisms were no more competitive with indigenous bacteria than was $PR1_{23}$. TOM_{31c} was then transferred to an environmental isolate chosen as a dominant heterotroph (as judged by its predominance over other strains in unselected Wichita aquifer material plated to R2A or their demonstrated

environmental (i.e., amended aquifer material) dominance: WS23 (TOM_{23c}) was tested for *in situ* degradation of TCE in soil columns and contaminated aquifer material at NAS Whiting Field (Florida). Results indicate a definite difference in the TCE-degradative capacity of these strains under these simulated aquifer conditions.

G. CONCLUSIONS

1. Direct, one-time application of derivative G4 strains to bioreactors or environmental matrices does not lead to effective degradation of TCE in either liquid or vapor-phase.

2. G4 strains do not form an extensive biofilm on any of the support matrices examined, despite its ability to colonize oyster shell to a significant extent under highly buffered conditions.

3. The single greatest weakness seems to be the inability to directly select G4 strains for the constitutive cometabolic activity, which is the only phenotypic marker that differentiates them from the wild-type bacteria, and for that reason the only one that matters in these applications.

4. TOM contains the entire Tom operon, and, because it is a self-transmissible plasmid, it is possible to transfer the Tom constitutive expression phenotype to other bacteria by simple bacterial conjugation. This has allowed the creation of other strains expressing this critical Tom constitutive phenotype, that are far better biofilm producers than G4 strains: JS150 (TOM_{31c}) and 249 (TOM_{31c}).

5. The newly created biofilm producing, Tom constitutive strains while capable of forming a highly active biofilm were not competitive with indigenous bacteria under the selective conditions tested.

6. Alginate and polyurethane encapsulation/entrapment methods proved ineffective in retaining $PR1_{23}$ for bioreactor applications. The alginate polymer was too fragile and the polyurethane did not retain the bacteria.

H. RECOMMENDATIONS

Any successful application of the Tom constitutive phenotype (or any other TCE cometabolic phenotype) will first require one of two prerequisites. An engineered environment that protects the host bacterium from environmental pressures of competition and predation and that will allow it to survive at functionally significant concentrations (i.e. $> 10^8$ cells/ml) (i.e. encapsulation), or a host that can tolerate these negatively selective pressures while degrading TCE. Selective manipulation of alternate bacterial hosts to provide more environmentally stable strains is possible because Tom is encoded by the mobile plasmid TOM, which we now know is expressible in other bacteria.

The enzyme Tom can function effectively under environmental conditions was shown; this clearly points the way to strain selection and improvement.

The results from Whiting field, while somewhat confounded by indigenous TCE-degradative capacity, nevertheless indicate that application of the *in situ* recirculation/bioaugmentation process as modeled there, should result in the complete degradation of BTEX and chlorinated ethene contaminants at this site.

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SECTION I

INTRODUCTION

A. OBJECTIVES

The primary goal of this investigation was to determine the suitability of bacterial strains developed through genetic manipulations (Shields and Reagin, 1992) for the cometabolic oxidation of trichloroethylene (TCE) (and several related isomers) under field conditions. This required development of treatment scenarios in the laboratory to be tested in the field, where flawed process components were identified for further laboratory examination and possible correction.
The objectives of this proposal are basically twofold:

1) Evaluation and characterization of the ability of this bacterium to colonize bioreactor support materials that will allow it to function as a gas or liquid phase biofilter for the destruction of air or water entrained TCE. This involved laboratory determinations of the biological stability of the strains, bioreactor requirements for activity and maintenance, substrates treatable in air/liquid streams, and the physical parameters affecting the efficiency of this process and effects on the physiological state of the bacteria.

2) Development of a testable *in situ* application of these genetically altered strains. The capacity of the strains with which the study was started, and those developed during the course of the investigation, were assessed for their stability in nonsterile soils, requirements for coreductants, *in situ* degradation kinetics, and usefulness in a plausible treatment system.

B. BACKGROUND

1. Statement of the Problem

Groundwater contamination by organic pollutants is of overwhelming concern to the industrialized world. Volatiles represent the most frequently detected group of groundwater priority pollutants. Some of the most often encountered components of this largest pollutant class are TCE, perchloroethylene (PCE), *trans* -1,2-dichloroethylene (DCE), and 1,1-DCE (ranked first, second, third, and fifth, respectively, of all volatiles detected (Rajagopal, R. 1986. Environ. Prof. 8:244-264)). The most prevalent of these, trichloroethylene (TCE), has become the focus of many studies on its biodegradability. TCE remains a persistent aquifer contaminant due to its relatively high water solubility (approximately 8 mM @ 25°C), density, its failure to partition to soil and aquifer matrices. This allows rapid entry into aquifer systems where it collects as dense non-aqueous-phase liquids (DNAPLs), and slowly bleeds off into the aquifer stream as a pollutant

plume. TCE remains very persistent underground, where it is often not subject to chemical or biological transformations under existing conditions.

2. Bacterial Degradation of TCE

Despite the exceptionally low rate of transformation of TCE by microorganisms in the environment there have been numerous reports of bacteria capable of the metabolism of TCE and related isomers. All reports of TCE transformation by anaerobic bacteria indicate a very slow process (Kleopfer et al., 1985) (Barrio-Lage et al., 1988) (Bouwer, Rittmann, and McCarty, 1981) (Bouwer and McCarty, 1983) (Freedman and Gossett, 1989) which is often associated with the production of vinyl chloride a known carcinogen (Vogel and McCarty, 1985).

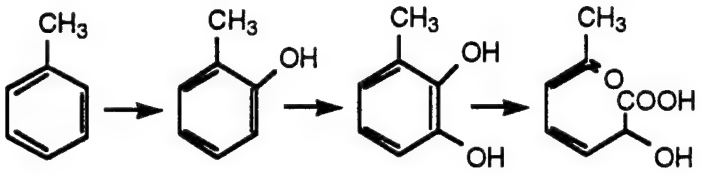
The relatively rapid mineralization of TCE by aerobic bacteria has been demonstrated for several isolates. All require the addition of an exogenous chemical inducer for the production of the requisite TCE degrading enzymes except *Nitrosomonas europeae* (Arciero et al., 1989) (Vannelli et al., 1990) which requires ammonia as a coreductant. *Pseudomonas putida* F1 (Nelson et al., 1988) (Wackett and Gibson, 1988), *P. mendocina* (Winter,Yen., and Ensley, 1989), and *Burkholderia (Pseudomonas) cepacia* G4 (Nelson et al., 1986) (Nelson et al., 1987) all require the addition of toluene or phenol to induce TCE degradative enzymes. Two methanotrophic bacteria: *Methylosinus trichosporium* OB3b (Oldenhuis et al. 1989) and isolate 46-1 (Little et al., 1988) have been reported to degrade TCE following stimulation of TCE degrading enzymes with methane. A derivative of G4: $PR1_{23}$ was developed in my laboratory and is constitutive for *ortho*-toluene monooxygenase (Tom) (the enzyme responsible for TCE oxidation) through the introduction of the transposon Tn*5*. Subsequent rearrangements of the organism's genome led to the constitutive production of Tom.

3. Envisioned Role of a Constitutive TCE Degrader in Aquifer Remediation.

Current technologies for the treatment of TCE contaminated soil and water have relied primarily upon pump-and-treat systems whereby TCE is distilled away from the water under vacuum, is air-stripped and transferred, or is sorbed directly, onto an adsorbent such as charcoal. In either event, the end result is simply the production of more polluted material or atmosphere. The capability to destroy the contaminant at the site would represent significant environmental and economic benefits. If realized, Bioremediation technologies would fill this need.

4. Biochemistry

Toluene catabolism by G4 has been shown to proceed via a monooxygenation pathway that results first in an *ortho* -hydroxylation of toluene and subsequently an *ortho* -hydroxylation of the resultant cresol to form 3-methylcatechol (Shields et al., 1989).



This pathway is utilized for growth on toluene, phenol, benzene, cresol, and xylene isomers by G4. Studies of various mutants of this pathway have revealed that the function required for the hydroxylation of toluene and phenol is also that required for the oxidation of TCE (Shields et al., 1991). The limitations of these bacterial systems for the bioremediation of TCE in the environment are that all natural isolates degrade TCE fortuitously. Their ability to alter TCE is necessarily linked to the production of an enzyme that can coincidentally accept TCE as an alternative substrate, the native substrate being that which is used to induce its synthesis. Due to this cometabolic relationship TCE cannot be degraded in the environment without the addition of an inducing substrate. As a result these organisms are faced with the task of degrading TCE only when in the presence of an inducing substrate that must compete for the same active site on the induced enzyme. In addition this also means that the organisms are not active beyond the environmental zone that can be controlled through the addition of effective concentrations of inducer: i.e. the bacteria are essentially "tethered" to the inducing substrate. Both of these limitations have serious implications to the design of both environmental and bioreactor applications. In addition the application of the native inducing substrates such as toluene or phenol is not possible in the environment as these are pollutants themselves.

The relatively rapid mineralization of TCE by aerobic bacteria has been demonstrated for several isolates, all of whom require the addition of an exogenous chemical inducer for the production of the requisite TCE degrading enzymes (except *Nitrosomonas europeae* which requires ammonia as a coreductant).

Since bacterial cometabolism of TCE only occurs fortuitously, the ability of bacteria to alter TCE is necessarily linked to the production of an enzyme that can coincidentally accept TCE as an alternative substrate, the native substrate being that which is used to induce its synthesis. Due to this cometabolic relationship TCE cannot be degraded in the environment without the addition of an exogenous inducing substrate. As a result these organisms are faced with the task of degrading

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5. Development of a constitutive TCE degrader

Transposon-induced mutants of *B. cepacia* G4, unable to grow with phenol or toluene or degrade TCE (strain G4 5223 and 5231) were found to revert to phenol utilization through the constitutive expression of Tom ($PR1_{23}$, and $PR1_{31}$). These strains were found to be genetically stable through at least 100 generations of nonselective growth and, without induction, capable of expressing Tom and catechol-2,3-dioxygenase at 50-100% of the wild type fully induced levels (Shields and Reagin, 92), and consequently degrade TCE without exogenous aromatic inducers. Such strains are attractive as biodegradative agents for the remediation of groundwater contaminated with TCE. Neither $PR1_{23}$ or $PR1_{31}$ contain recombinant DNA sequences. Manipulations were through genetic techniques normally encountered in the environment, and the only "foreign" DNA is that of TN*5* a transposon known to exist in the environment, and is under no known barrier for natural transfer to strains like G4.

Experiments were carried out to determine the extent of TCE removal by these constitutive strains. 94 - 99.9% degradation was seen in simple degradation assays with untreated soil and sand slurries. The capacity for $PR1_{23}$ to colonize a solid support matrix, and subsequently degrade TCE was assessed for six materials including glass beads, sand, gravel, charcoal, diatomaceous earth pellets and oyster shell. Oyster shell provided the best overall performance characteristics. In addition to providing the highest activity towards TCE in the degradation assay (i.e. 100% degradation) it has the added potential advantage of buffering the HCl produced from the metabolism of TCE, since it is composed almost exclusively of $CaCO_3$.

6. Genetics

Hybridization analysis of Tn*5* mutants of G4 lacking Tom activity, suggested that this capability is encoded by the largest (~109 kb) resident plasmid of this strain, TOM_{23c}. This is a new toluene degradative plasmid completely unrelated to the archetypical TOL (Shields et al., 1995).

C. SCOPE

1. Phase I: Field trial of bioreactor support matrices at Hanscom AFB

This phase involved the immediate progression to the field with our preliminary understanding that a biofilm of $PR1_{23}$ could be established in the laboratory on a crushed oyster shell support matrix. The purpose in the field was to determine if this could be repeated under field conditions as well as to identify problems with this anticipated mode of application and to field test monitoring protocols developed in the lab.

Two sites were examined: Hanscom and Wurtsmith AFB. Of the two, Hanscom was judged to have superior qualities for our needs. These included an autoclave site, and much more help in interfacing with an existing air stripping tower. The facility at Wurtsmith, while comparable in the construction and access was complicated by water being chlorinated before introduction to the air strippers. In addition the location of the Wurtsmith facility guaranteed substantial delays in parts acquisition and replacement.

2. Phase II: Encapsulation, entrapment and alternate biofilm strain development.

Phase I taught that a primary limitation of the biodegradative strains developed thus far was a lack of competitiveness under environmental conditions. In view of the cometabolic nature of the application there was little possibility that this could be approached directly. Genetic methods were employed to attempt to produce a more aggressive colonizer of the bioreactors, that was still constitutive for TCE degradation. Also, alternate approaches for maintaining $PR1_{23}$ in a liquid phase bioreactor were also undertaken. These included alginate encapsulation and membrane immobilization.

3. Phase III: *In situ* recirculation well application of $PR1_{31}$ modeled at NAS Whiting Field

The application of $PR1_{23}$ and two alternate constitutive TCE degraders (produced during phase II) to an *in situ* treatment scenario was attempted, for two reasons. The major reason was that an *in situ* groundwater recirculation device could be modeled above ground as a soil core, and this represented a logical route of delivery. The other reason is that inoculation at a high enough density may allow the continued degradation of TCE at moderate to low input levels of a cometabolic carbon and energy source. In this way, it may not be necessary to demand competition during a colonization and growth phase.

SECTION II

PLUGGED FLOW BIOREACTOR APPLICATIONS OF *Burkholderia* (*Pseudomonas*) *cepacia* $PR1_{23}$

A. INTRODUCTION

The first phase of this study was to develop a bioreactor system capable of TCE degradation from contaminated air or water utilizing strain $PR1_{23}$. Preliminary laboratory experimentation was conducted to identify a bioreactor design, solid support matrix and methods for determining the effectiveness of bacterial colonization and activity relative to the target enzyme: toluene *ortho*-monooxygenase (Tom).

During the development phase of the biofilter bioreactors Hanscom AFB was selected over Wurtsmith AFB as a more optimal trial site. Hanscom is an active base with better access to fundamental support (electrical and autoclave facilities primarily), and the facility itself was staffed 24 hrs. In addition the proximity of the Boston area assured easier access to materials and supplies.

B. METHODS AND MATERIALS

1. Organisms and Culture Conditions

The only organism utilized in this phase of investigation for the degradation of TCE was *B. cepacia* $PR1_{23}$. Unless otherwise noted it was routinely cultured on basal salts medium (BSM *see below*) with 20 mM lactate as the sole carbon source.

2. TCE Degradation Analyses

Standard Bottle Assay for TCE Degradation: TCE degradation was determined following an overnight incubation of washed cells suspended in 2 mL of BSM containing 20 μM TCE (unless otherwise noted) (calculated as though all were in solution) in a 12 mL glass vial, sealed with a Teflon™ lined rubber septum. Equal volume pentane extracts were performed to determine residual TCE concentration by gas chromatography using an electron capture detector (Shields et al 1989).

Rates of TCE degradation were determined using cell suspensions in a 50 ml glass syringe with a Teflon™ plunger without an air headspace as previously described (Folsom et al 1990). This assay allowed multiple non-destructive sampling (1 ml each).

The decision was made to utilize an existing gas chromatograph and purge and trap device from Tyndall with the following modifications: Addition of an auto-sampler and control unit that will interface with the existing integrator, with a VOCOL column.

3. Media

Basal salts medium (BSM) (Hareland et al. 1975) was originally formulated for the growth of hydrocarbon utilizers in a defined carbon free matrix. However because it was derived primarily from stocks used for enzymological work it was based on a very strong phosphate buffer system. It contains the following components at pH 7.2 (in g/L water): $K_2HPO_4 \cdot 3H_2O$ (4.25), $NaH_2PO_4 \cdot H_2O$ (1), NH_4Cl (2), Disodium salt of nitrilotriacetic acid: $[HO_2CCH_2N(CH_2CO_2Na)_2]$ (0.12), $MgSO_4 \cdot 7H_2O$ (0.2), $FeSO_4 \cdot 7H_2O$ (0.012), $ZnSO_4 \cdot 7H_2O$ (0.003), and $MnSO_4 \cdot H_2O$ (0.003).

4. TFMP Oxidation Analysis for Tom Activity

One of the products in this section is the development of this assay. The A_{600} of the culture is recorded. 1 ml is pelleted in a microfuge (15,000 rpm for 30 seconds) and resuspended in 1ml of 10mM Tris-Cl pH 8.5 1.0mM triflouromethyl phenol (TFMP) (also: m-hydroxy benzo triflouride). These cells are then incubated in a 25ml Erlenmeyer flask at 30°C for 20 min open to the air. The process to this point can be used as a qualitative assay for yellow color development (i.e. triflouroheptadienoic acid (TFHA) production [molar extinction coefficient at 385 nm = 26,900 AU.L/mole]). In order to quantitate the enzyme activity the cells are again pelleted in a 1.5 ml microfuge tube, 15,000 rpm/ 30 seconds. The supernatant is transferred to a clean tube and place on ice until ready to read. Care must be taken to avoid any cellular material. The A_{386} and A_{600} of each cleared supernatant sample is recorded. Since the yellow TFHA product absorbs strongly at 386 but not at 600 nm the A_{386} absorption actually due to light diffraction of whole cells can be subtracted by measuring the ratio of A_{386} /A_{600} (using a low concentration of cells: where the A_{600} is < 0.1). This allows conversion of the A_{386} measured to a corrected A_{386}.

$A386_{corrected}$ = $A386_{measured}$ - $A386_{due\ to\ diffractive\ loss}$

= $A386_{measured}$ - [($A600_{sample}$) x (C)]

Where C is the A_{386} /A_{600} ratio .

Calculations:

mg Protein/ml (for G4 strains) = A600 x 0.290

micromolar TFHA = A386 corrected/ 0.0269

nmoles TFHA = μM x 0.001L x 1000 nmol /μmol

nmoles TFHA/min = nmol TFHA/20 min

Specific activity: nmoles TFHA/min/mg protein

5. Biofilm Measurement

To quantitate the biomass associated with a biofilm, $PR1_{23}$ cell associated protein concentrations were determined by the BCA method, standard incubation protocol, (Pierce Chem. Co., Rockford, IL) with bovine serum albumin as the protein standard, in the presence of crushed oyster shell.

Standard curves for A_{600} and the associated protein content of LB and phthalate grown cells were established. Estimates of protein content based on A_{600} measurements were made with both LB and phthalate grown cultures based on extrapolation from these curves. Such measurements however were not helpful with the oyster shell associated biofilm, since any attempts to resuspend this material results in a fine 'flour' with substantial 600 nm light scattering properties as well.

C. RESULTS

1. Laboratory Studies

a. Carbon Sources for Biofilm Development.

Experiments were initiated on the growth of $PR1_{23}$ on various carbon sources. These included lactate, citrate, phthalate, pyruvate, glucose, yeast extract, dried bacterial extract, and soluble and insoluble starch.

(1). Growth Curves

Studies were done at both 15 and 30°C. There was no qualitative difference between utilization of any of the nutrients tested at the two temperatures. The more complex carbon and energy sources gave rise to more immediate growth, followed by a more linear growth phase. These characteristics are seen as advantageous under conditions of high substrate concentration and oxygen limitation. A possible disadvantage is that complex carbon sources may be considerably less selective.

Lactate and citrate yielded moderate cell mass, with much reduced (as compared to 30°C) log growth rates at 15°C. Bacterial extract (prepared from dried, autoclaved, bacterial pellets) gave the longest term growth with the least increase in cell mass. Because of the obvious chemical similarities between bacterial and yeast extract as growth substrates, the findings of a much more linear growth on bacterial extract at 15°C is somewhat surprising (note that the growth curves for both yeast extract and bacterial extract were performed twice at 15°C {i.e. Y1 & Y2 and B1 & B2 respectively})

Phthalate was found to be a generally useful carbon and energy source for the routine cultivation of $PR1_{23}$ in the laboratory. Pyruvate and phthalate allowed growth at rates similar to those seen with lactate. Phthalate and lactate demonstrated a higher cell yield which closely approached that achieved with yeast extract.

No growth was seen with the two starch substrates.

(2). Specific Activities

Specific activities of batch cultures at various concentrations of lactate and phthalate were measured over a 48 hour period. Overnight cultures of lactate or phthalate grown cells were

pelleted and resuspended in various concentrations of the same nutrient again, and monitored for specific activities of TFHA production.

The specific activity of lactate grown cells, suspended in BSM containing 1.0, 0.1, 0.01 mM or no lactate was found to be ~2.5 nm TFHA/min/mg protein overall for all treatments after 48 hours. 10 mM supplemented BSM gave the lowest recorded activity (~0.4 nmoles TFHA/min/mg protein) (Figure 1).

This same experiment was performed with phthalate as the sole carbon source and specific activities were monitored throughout the same 48 hour period. The cells were suspended in either 20, 2.0, 0.2, 0.02, or 0.002 mM phthalate in basal salts medium. The general trend remained the same as for the lactate-fed cells. At the lower phthalate concentrations, the specific activity of TFHA production stabilized at the same general specific activity as the lactate-grown cells: ~2-3 nm TFHA/min/mg protein. The culture resuspended in 20 mM phthalate quickly became unresponsive to TFMP (Figure 2).

Several carbon sources were identified that should satisfy the metabolic requirements of $PR1_{23}$ during the co-oxidation of TCE. They have been found to support the growth of $PR1_{23}$, TFMP oxidation, and some support biofilm development.

The behavior of the lactate- and phthalate-starved batch culture cells was very interesting. In general, a trend towards low-level stable expression of the *tom* operon without additional input of nutrients was obvious. This may be a positive attribute when considering the necessity to not overfeed the bioreactors, especially under conditions where we wish to introduce a contaminated water stream. It may also suggest a nutrient feed scenario in which the nutrients may be pulsed and not continuously fed. This could have profound ramifications in the area of preventing invasion of the biofilm by non-TCE-degrading bacteria.

The reason for the loss of activity under high nutrient concentrations remains unexplained, but it is likely peculiar to batch culture conditions.

(3). Starvation responses

(a) Survival : Lactate Grown Cells

A prolonged starvation experiment, using lactate grown cells, was performed at 30 °C in shake flasks containing 1, 10, 100 and 1000 mg/l lactate in basal salts medium. Cell survival was monitored as total viable counts on LB agar (Figure 3).

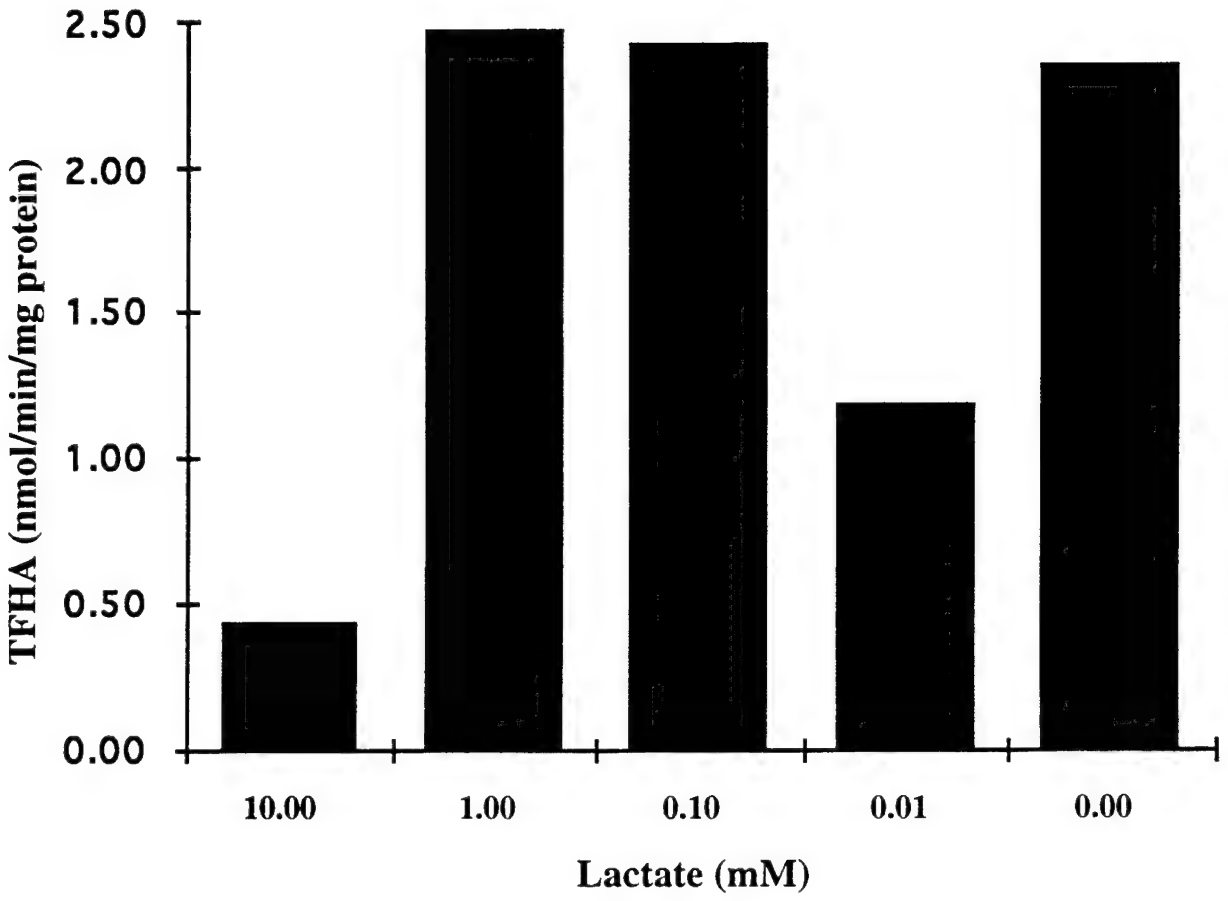


Figure 1. TFHA Specific Activity from a 48 hour Batch Culture of $PR1_{23}$

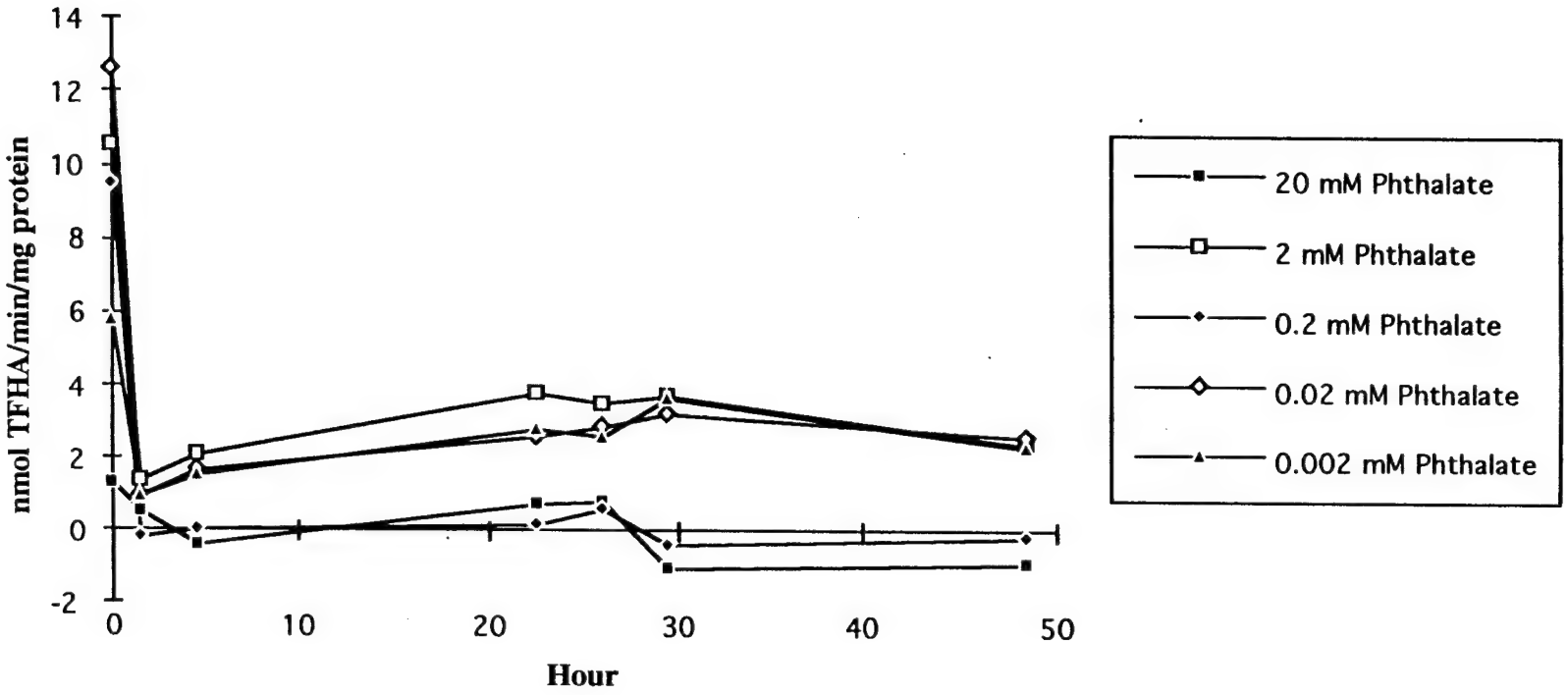


Figure 2. Phthalate concentration effects on TFHA Production by $PR1_{23}$

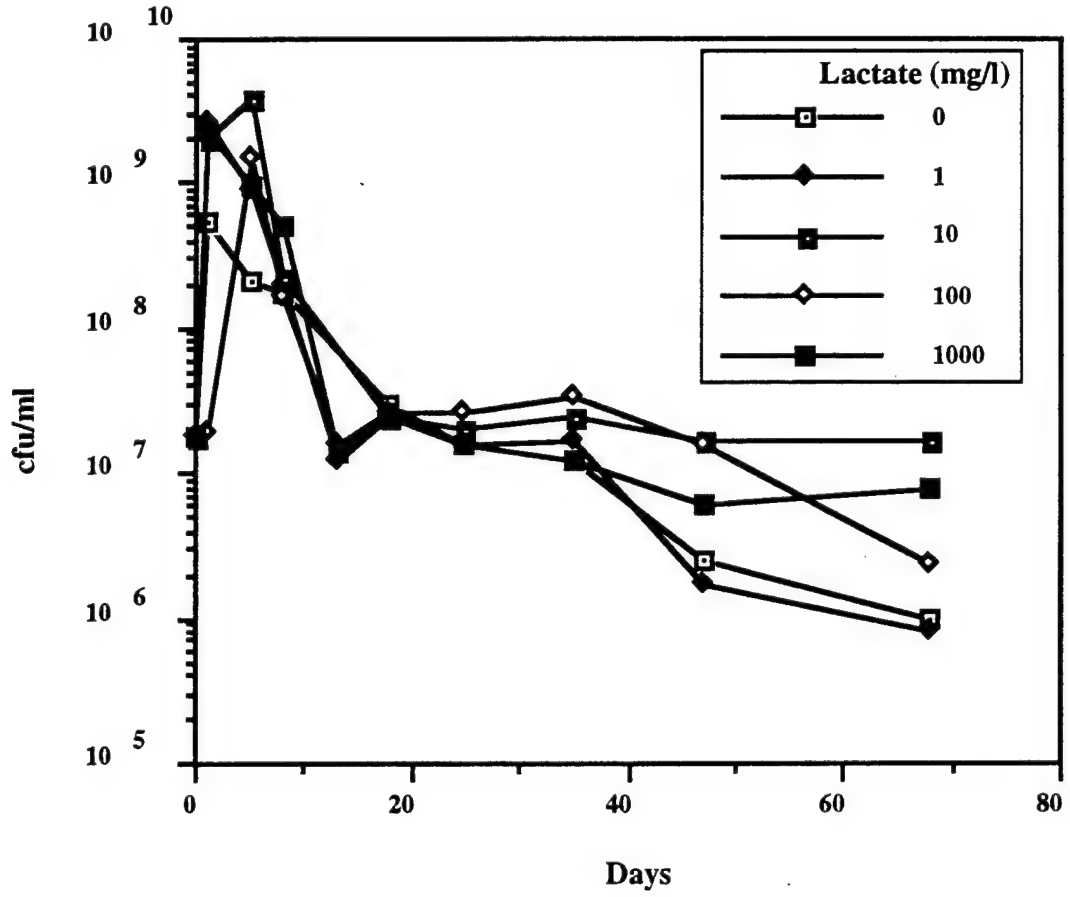


Figure 3. $PR1_{23}$ Starvation Following Lactate Growth.

Survival of lactate grown cells was measured from 500 ml flasks containing 100 ml of medium shaking at 100 rpm. A maximum growth corresponding to ~2-3x10^9 cfu/ml was attained for all flasks, regardless of lactate concentration. All cultures attained maximal TVC by 24 hours, and maintained this for the next 24-48 hours. Culture optical density did not decline proportionally through the test period (Figure 4), indicating a genuine decrease in individual cell viability.

(b) TFMP and TCE oxidation activities: lactate and phthalate

Short term starvation

Experiments were initiated to determine the effects of starvation on Tom activities and to determine if the relationship between TCE and TFMP oxidation rates would remain high. This would answer how good the more easily measured rate of TFMP oxidation would be at predicting the TCE degradation rate. Since lactate and phthalate were shown to provide an adequate nutritional base for $PR1_{23}$ in basal salts medium (BSM), it was of interest to determine what rate of cell feeding would be required to maintain activity. To address this a $PR1_{23}$, grown in batch cultures, in 10 mM phthalate or lactate were pelleted, washed and resuspended in 0.001 mM of their respective growth substrate in BSM. The specific oxidation activity of these cells towards TFMP were then monitored daily for 6 days. The viable numbers of $PR1_{23}$ were determined from 30°C shake flasks over 25 days by spreading dilutions to LB or LB kanamycin plates and growing colonies at 30°C.

The specific activity of the lactate and phthalate starved cells measured over this period is presented in Figure 5.

Triplicate 2 mL samples from this assay were tested for their ability to degrade TCE in a standard bottle assay (i.e. standard TCE assay performed in inverted bottles with Teflon™ lined rubber septae containing an air headspace) with TCE at 20 μM (calculated as though all TCE were in aqueous solution, when in fact this concentration depends on many factors since TCE has the propensity to exist in the vapor phase as well (i.e. due to its Henry's law behavior). Samples were assayed on successive days beginning 24 hours after the inoculation of the phthalate culture. A parallel culture of $PR1_{23}$ on 20 mM lactate, sequentially transferred to fresh media each day, was run as a positive control. Abiotic

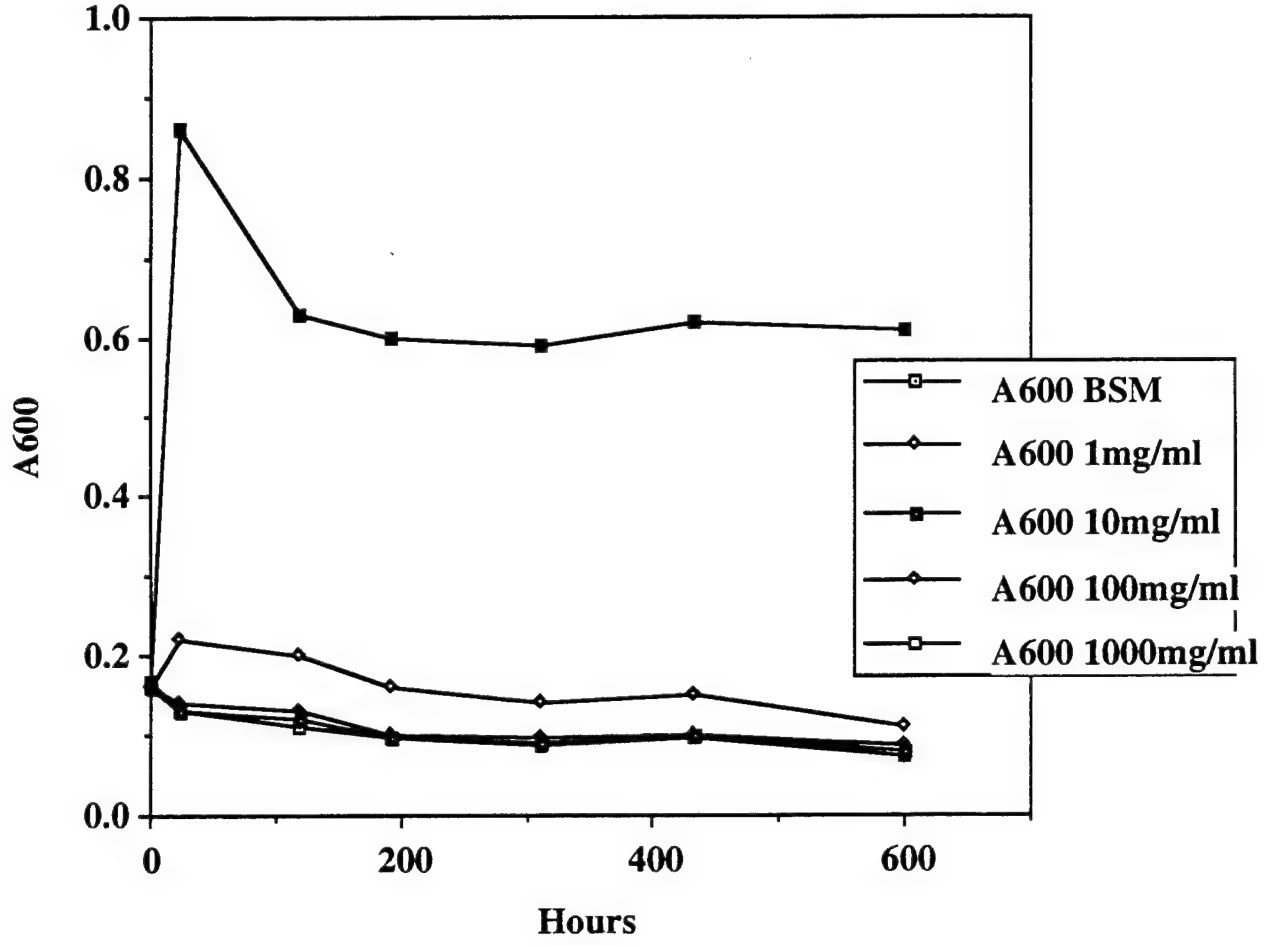


Figure 4. Starvation Effects on Cell Mass Following Lactate Growth of $PR1_{23}$

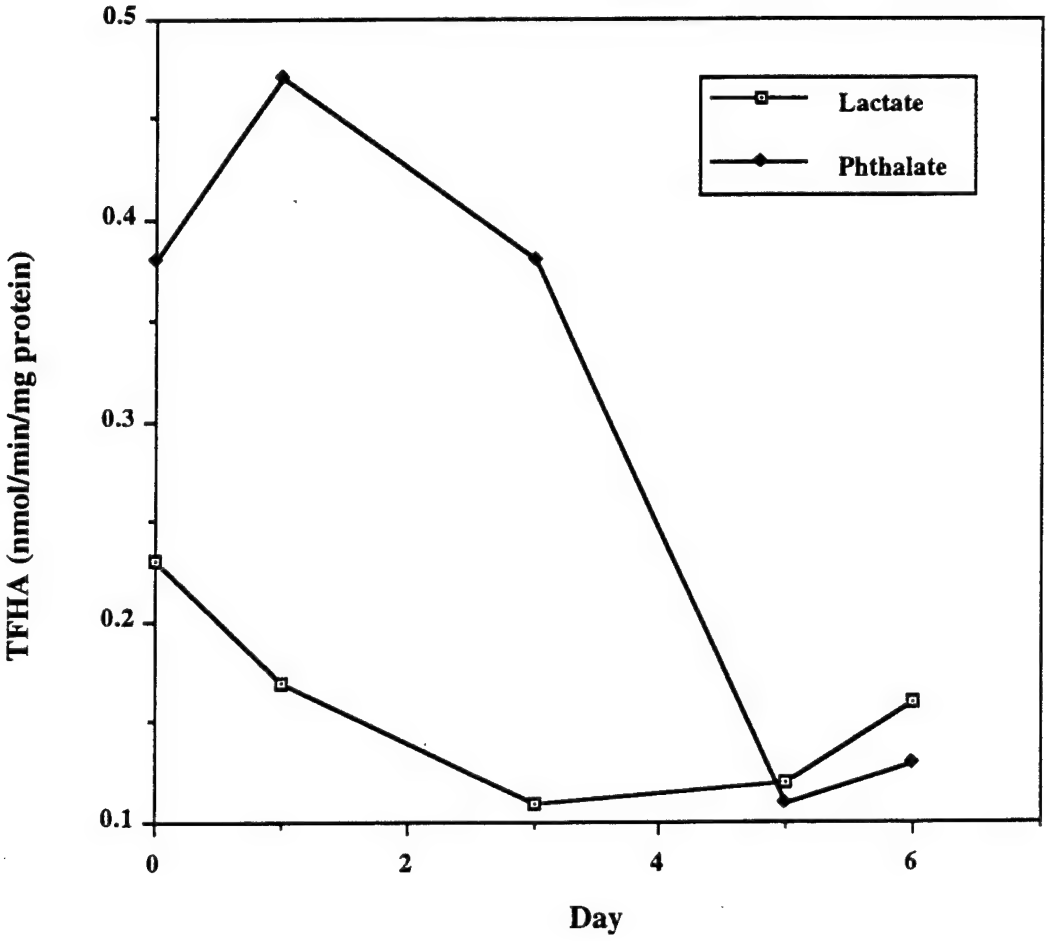


Figure 5. Starvation Effects on TFMP Oxidation Rates by $PR1_{23}$

controls containing BSM only were run as well. The results of this experiment are given in Figure 6, and indicate the TCE degrading capacity of phthalate grown $PR1_{23}$ is not affected during at least the first 53 hours of starvation.

Long-term starvation. The specific oxidation activity of $PR1_{23}$ grown on either lactate or phthalate (10mM)towards TFMP were reported for a period of 6 days after washing these cells and resuspending in carbon free medium. The phthalate fed experiment was continued through 36 days, with periodic pulses of increasing phthalate concentration. The purpose was to determine the continued expression Tom activity by $PR1_{23}$. Starvation effects as measured by TFMP oxidation in response to phthalate starvation and readdition to a static culture is presented in (Figure 7). The ability of $PR1_{23}$ to survive starvation conditions appears to be very good. Both lactate and phthalate appear to affect $PR1_{23}$ in a similar manner. Perhaps the most surprising finding is the capacity of $PR1_{23}$ to maintain TFMP oxidation activity for so long (6 days) after all utilizable carbon (save that available through cell turnover) is absent. Subsequent experiments were conducted to ascertain that the residual oxidation activity seen, 0.1-0.16 nmol TFHA produced per min per mg protein, was indeed a function of Tom pathway enzymes.

It appears that continual feeding of the $PR1_{23}$ biofilm may not be necessary, nor even desirable, as continual feeding would only encourage contaminant growth. Pulse feeding would therefore be not only an efficacious but preferable option.

The ability of $PR1_{23}$ regain TFHA oxidation potential (after starvation the point on non-detectable activity) was rapid and proportional to the concentration of phthalate to which the cells were exposed. Maximal activity would seem to lie somewhere between 10 and 2 mM phthalate, although higher concentrations of phthalate were not investigated. This data would support a field strategy of pulsed feeding of phthalate to the bioreactors on a daily basis, for brief periods at high concentrations. Less surprising, the capacity to oxidize TFMP must be seen as conservative measures of TCE degradative capacity only.

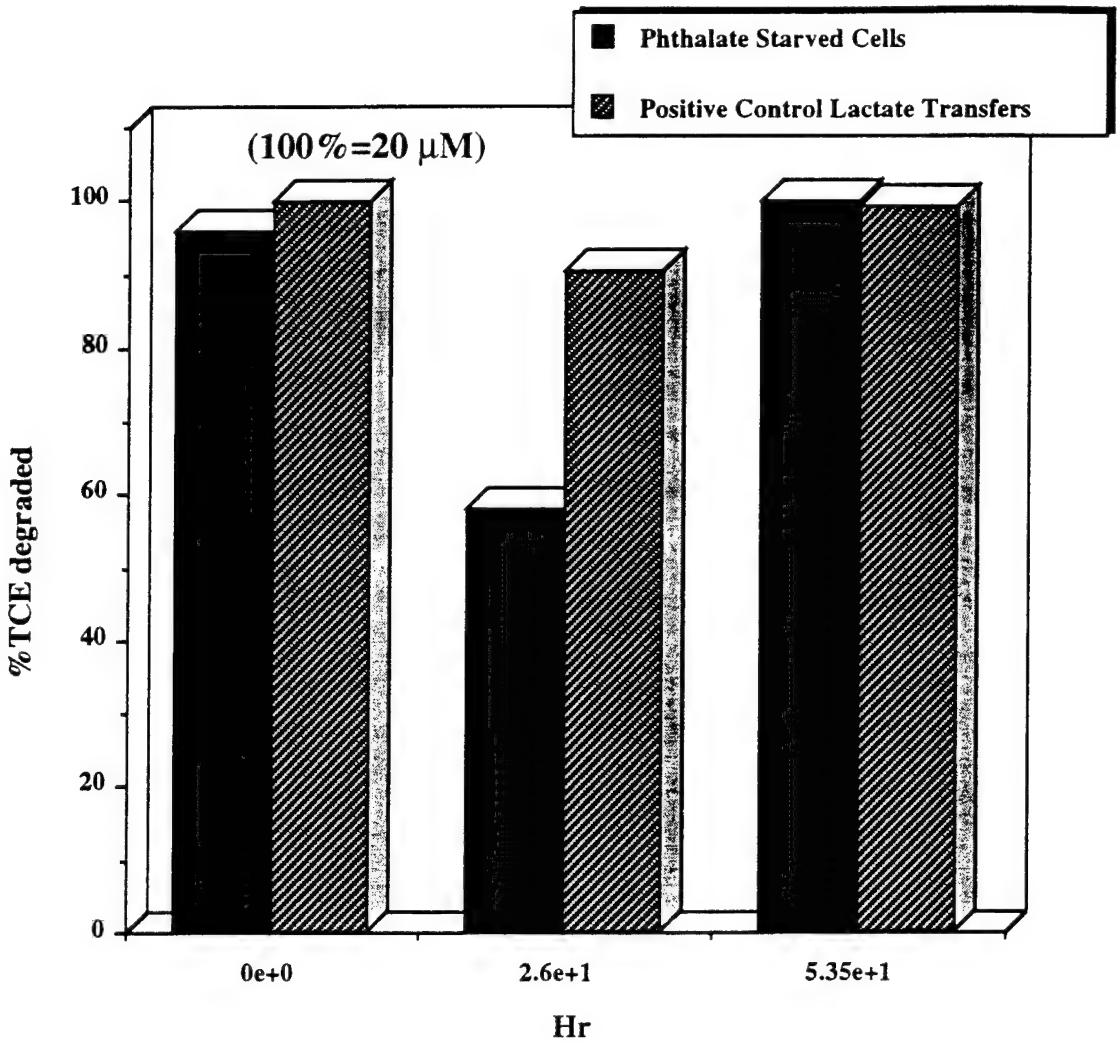


Figure 6. TCE Degraded in Overnight Assay

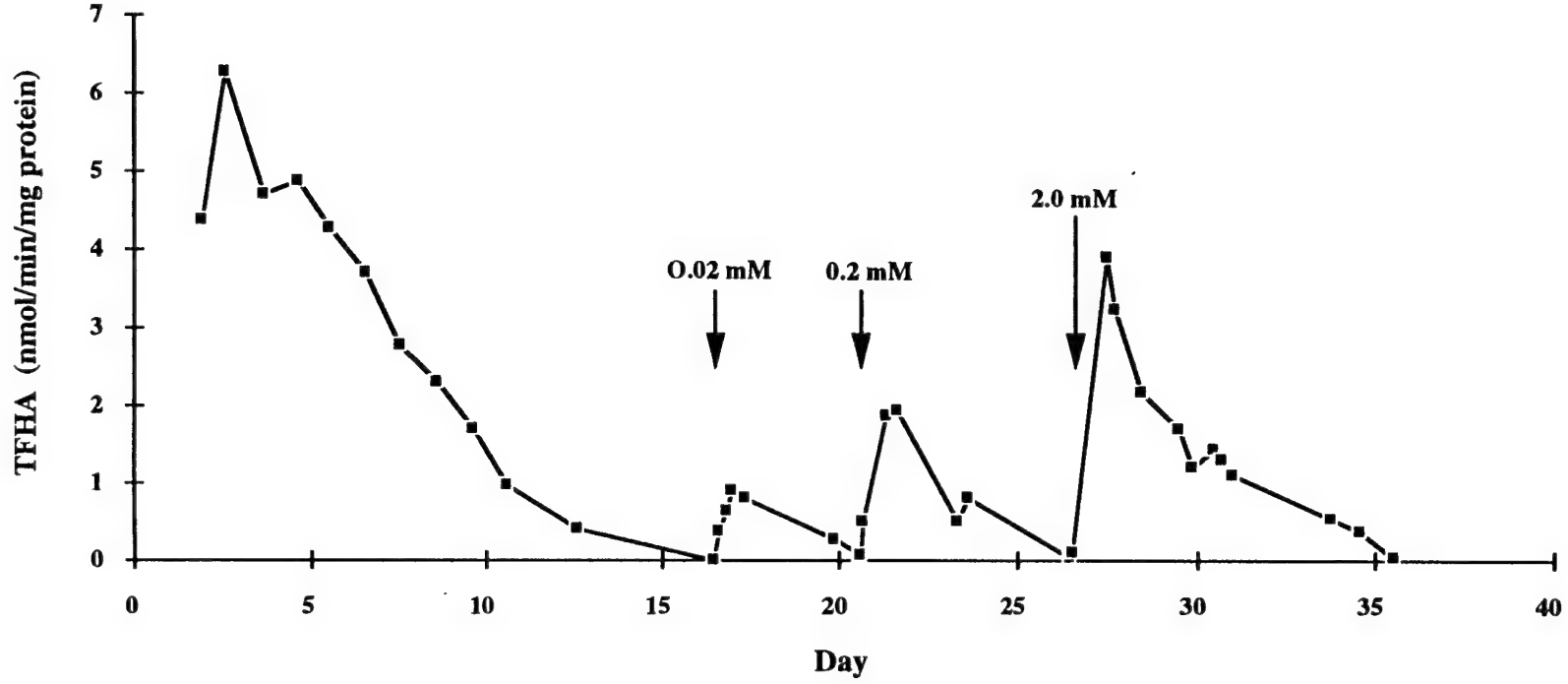


Figure 7. TFHA Specific Activity in Response to Phthalate Addition During Prolonged Starvation of $PR1_{23}$

(c) Small Laboratory-Scale Oyster Shell Columns:

(1) Colonization of a non-volatile carbon source

Small-scale reactors, constructed of 50 cc glass syringes, were used to determine the rate and extent of oyster shell colonization as a function of recirculation Rates, nutrients (carbon: Lactate, Phthalate, Phenol, and yeast extract; inorganic: O2 {including peroxide}, NO_3 and PO_4), nutrient feed rates, and methods of Inoculation over a 2-week period. Phthalate, phenol and the no-carbon columns were the only ones that did not demonstrate significant contamination. Of these only the phthalate fed columns seemed to colonized effectively (based on recirculating total viable count).

50-cc recirculating reactors (Figure 8) were fed basal salts medium (no carbon source), phenol, phthalate, or lactate at 2 mM or yeast extract at 0.3% for two weeks. The recirculating fluid was periodically sampled and the total viable count, and % TFMP positive colonies determined. Care was taken to ensure sterility of the reactors before inoculation but this proved impossible to maintain for long due to separation of various tubing connections. The column materials have still to be assayed for their protein content.

Cell counts of the recirculating fluid of the phthalate fed columns were shown to contain between 3 x 10^7 and 3.5 x 10^8 viable cells per ml throughout the nine day period. Phthalate maintained 93% (25/27)TFMP+cells through Day 8 of column operation, and 83% (15/18) by day 9. The lactate fed column on the other hand had decreased to 40% TFMP positive by day nine. It should be known that determination of TFMP capabilities of individual cells is dependent on colony size, and therefore the likelihood of false negatives exists where colony size is small, and it should therefore be viewed as a conservative measurement of % Tom positive. It is interesting to note that despite a reduction to only 8% (6/70) TFMP positive cells in the yeast extract fed columns, this value was back to 100% (10/10) the next day, and back down to 62% (5/8) on day nine. This kind of "yo-yo" fluctuation was not anticipated, and was not seen in the other columns (Table 1).

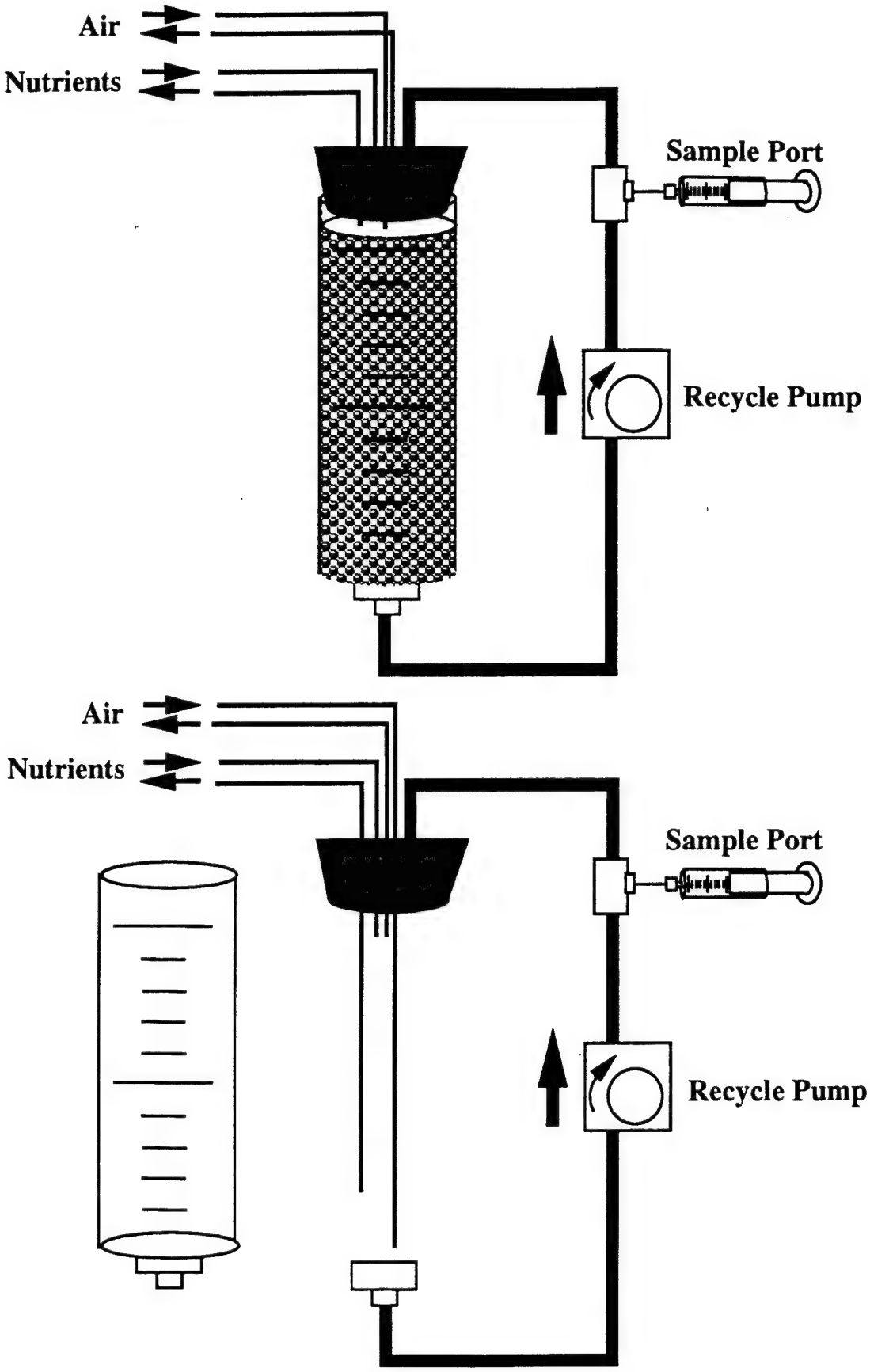


Figure 8. Minicolumn Diagram

Table 1 Activity of Effluent Cell Populations

Column	Hours	Viable Count x10^7	# TFMP+	% Colonies TFMP+
BSM	24	16	16	100
	48	3	3	100
Phenol	24	3	3	100
	48	13	13	100
Phthalate	24	12	12	100
	48	35	35	100
	120	3	3	100
	144	5	5	100
	168	27	25	93
	192	18	15	83
Yeast Extract	48	65	65	100
	72	77	76	98
	144	70	6	8
	168	10	10	100
	192	TNTC	0	
	214	8	5	62
Lactate	48	4	3	75
	214	5	2	40

Phthalate emerged as the most likely candidate for feed stock to maintain an active biofilm. It was both an adequate carbon source (reasonably inexpensive @ ~ $35.00/ kg Sigma Chemical Co.) and as a somewhat more selective growth agent than other carbon sources tested. This was supported by auxiliary experiments in which none of our other laboratory bacterial strains were able to utilize phthalate as a sole carbon and energy source: *P. putida, P. aeruginosa* , and *Alcaligenes eutrophus*, and *Acinetobacter* sp. (all representatives of likely contaminating bacterial groups).

Five of the six microcolumns (50 cc recirculating reactors) were tested for their ability to be successfully colonized by the direct application of batch-cultured (phthalate 10 mM) $PR1_{23}$ to columns receiving 0.02 mM phthalate at various rates of throughput. The recycling medium and oyster shell material were sampled and used to estimate the total number of colony forming units and their % TFMP+.

Each of the five columns was inoculated with 20 mL of an overnight batch culture of $PR1_{23}$ grown with 10 mM phthalate. All columns had been previously (within hours) equilibrated with 0.02 mM phthalate in BSM (i.e. the same formulation utilized for the batch culture, at 1/50 the carbon concentration). The relative rates of BSM phthalate delivery to each of the columns given in Table 2. Seven and ten days after inoculation all columns received an additional 1 and 20 ml, respectively, of an overnight (10 mM phthalate batch) $PR1_{23}$ culture. Samples from the recycle lines consistently gave small colonies at $>10^8$ cells/ml. The origin of these bacteria remains unknown, but difficulty in truly sterilizing the oyster shell is likely.

At 12 days, oyster shell sampled from these reactors gave unpredictable biofilm production and no detectable TFMP oxidative activity.

Table 2. TFMP Activity of Cells Cultured from Biofilm

Column #	Rate of 0.02 mM Phthalate Addition ml/min	CFU x 10^5	% TFMP+
1	0.11	No Growth	---
2	0.17	No Growth	---
3	0.32	>1,0000	
4	0.72	2.7	0
5	0.80	0.34	0

Continued sampling of the recycle lines failed to yield discernible $PR1_{23}$ colonies. This experiment was terminated at day 17.

Despite the ability of 0.02 mM phthalate to support TFMP oxidation activity in batch culture, it was far from optimal in aiding the establishment of a healthy biofilm of $PR1_{23}$. There have been substantial problems in getting a significant colonization of the oyster shell material as measured by turbidity measurements of the recirculating media of these small columns. A concentration of 10-20 mM phthalate, sufficient to yield an A600 value of ~2

after overnight shake flask culture at 30°C, fails to yield detectable turbidity even after 5 days at high recycle through the oyster shell, at similar temperatures. It was concluded from the above results that a much higher nutrient level might be more effective during the initial colonization phase.

Volatile carbon sources were used in microcolumns packed with oyster shell were used to determine the efficacy of a volatile organic carbon (VOC) for the establishment of an active biofilm. Toluene was determined to be the most efficacious route of carbon addition for the establishment of a heavy biofilm (i.e. >1 mg cellular protein per gram wet oyster shell) with high specific activity.

Toluene was not investigated as a carbon source for primary biofilm establishment earlier due to known problems through the competitive inhibition of TCE degradation. In the current circumstance this is not anticipated to be a problem, as toluene will only be tested for its fundamental utility in column colonization (see toluene vapor column diagram in Figure 9). In addition, feeding toluene in the vapor phase will mean a gradient in toluene concentration throughout the column as it becomes colonized. This means at the early stages the C:N ratio will be very high and remain so since the input of the carbon source is constant and not depleted significantly through metabolism. As seen from the glucose study above, the higher the C:N ratio the more successful the development of a biofilm.

Toluene was introduced to the microcolumns using an air-mixing system consisting of a 2 liter erlenmeyer flask containing a 20 mL vial of toluene through which the column air supply must first pass. Since the surface area of toluene exposed to the air stream was constant throughout the experiment, it was assumed that the concentration of toluene in the air stream (c.a. 7.6 mg per liter) remained relatively constant.

Sample of liquid from spent nutrients was plated and assayed for TFMP constitutive organisms. All cells were found to be constitutive for TFMP oxidation.

Colonization of the oyster shell with toluene vapor as the sole carbon source resulted in a rapid and heavy development of a high-activity biofilm with substantial microbial purity.

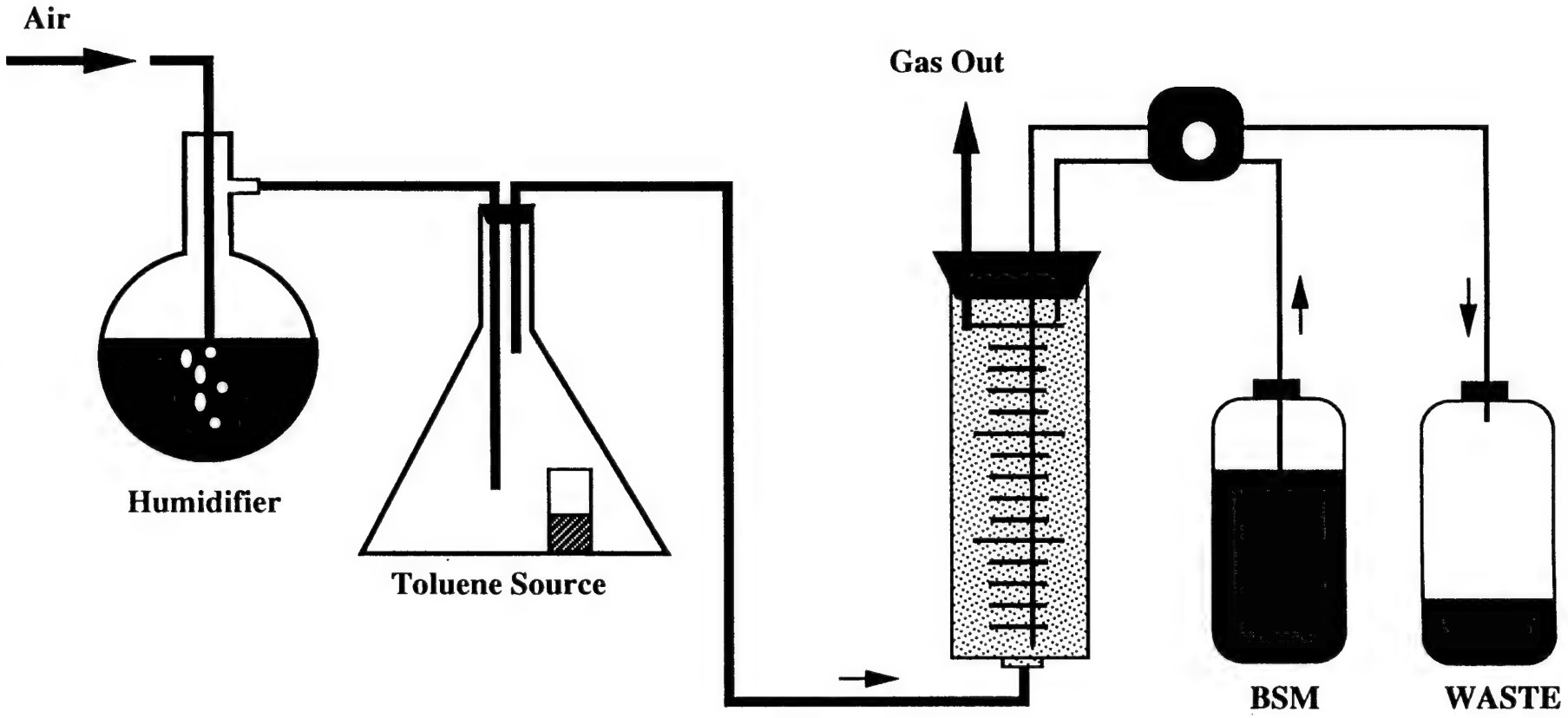


Figure 9. Toluene Vapor Column Diagram

(2). Effects of C:N ratios and carbon sources

We previously saw substantial problems obtaining a significant colonization of the oyster shell matrix within a minicolumn. These initial measurements were based on turbidity of the recirculating liquid. As such they may not be completely appropriate for biofilm determinations, since by its definition it remains attached. To test this oyster shell was wetted (fill and draw) and culture flasks were used for measuring biomass as a functions of: C:N ratios, and nonvolatile carbon sources: phthalate, glucose, and sucrose

To address the inability of 0.02 mM phthalate to support TFMP oxidative activity in an oyster shell biofilm, and the probability that other nutrients might be far more useful in the establishment of a primary biofilm, two experiments were initiated. One was to assess the affects of various C:N ratios on the ability of $PR1_{23}$ to successfully colonize the oyster shell surface in an active biofilm. The other was to determine if a vapor-phase organic such as toluene might aid in primary biofilm establishment.

Phthalate, glucose, sucrose and phthalate+glucose were tested for their ability to stimulate an adherent biofilm population on oyster shell. Instead of the flow through microcolumns this experiment was performed by twice daily rinsing inoculated oyster shell material in a shake flask with a modified BSM containing these carbon sources at 80 mM carbon. The amount of nitrogen (as ammonia) in the BSM in each case was varied to give C:N ratios of 100:1, 50:1, 15:1 and 2:1. Table 3 summarizes investigations to determine if the oyster shell itself affected our ability to measure protein associated with $PR1_{23}$. No effects were seen.

Table 3. Effects of oyster shell on protein determination

Sample	Protein (μg/ml)[a]
Shell alone[b]	6.28 ± 1.79
BSA alone	40.93 ± 0.45
BSA + Shell	43.37 ± 7.45
PR1 alone[c]	5868.97 ± 40.42
PR1 + Shell	5785.56 ± 25.30

[a]Determined via 70 oC/NaOH pretreatment method ± standard deviation of triplicate measurements

[b]Oyster shell present at 1 g/ml

[c]100 μl of an overnight PR1 culture assayed, and corrected to 1 ml.

There were no observed effects of the C:N ratios on TFMP specific activity, but there was a clear tendency to greater TFMP oxidative activity by the shell material at the higher C:N ratios when cultured in this manner. Glucose was the only carbon source clearly effective in the establishment of a significant biofilm. Part of the reason may be the short exposure time. This was completely intentional and was designed to encourage the growth of attached organisms. However, there was also undoubtedly an effect due the rapid transport of glucose by resting bacterial cells. Phthalate may not have been available during this brief exposure period (~30 minute-rinse) if specific transport systems had to be induced.

The major problem with these data is that they point to phthalate as being a poor material for establishment of a primary biofilm in the shortest time frame possible. Glucose, while effective in this regard has the added problem of undoubtedly encouraging invasion by other bacterial species during the colonization phase.

C. Optimization of the Rapid-TFHA Colorimetric Assay

A major goal was to design an assay of column function for TCE degradation by an indirect and rapid (i.e., within 1 hour) method. This was done by creating an assay capable of measuring the capacity of colonized reactor packing material (oyster shell) to convert trifluoromethyl phenol (TFMP) to its yellow (strongly absorbing at 388 nm) ring cleavage heptadienoic acid (TFHA) product (Figure 10). Previous tests were conducted in microfuge tubes containing cells resuspended in 0.5 mM TFMP, buffered to pH 7.0 in 10 mM Tris-HCl, and incubated uncovered (without agitation) for 30 minutes. After production of the soluble TFHA, the cells were removed by pelleting in the microfuge for 30 seconds. TFHA formation may be quantified through absorbance measurements at 388 nm (TFHA ε_{386} = 26,900).

The chemical parameters of the assay that defined the optimal conditions for TFMP oxidation were determined. This included pH, a vessel configuration that would provide maximum oxygen exchange during the reaction (but would still lend itself to multiple measurements under field conditions), temperature (the optimal temperature for TCE oxidation had

already been determined to be 30°C (Shields, 92), TFMP substrate concentration (necessary not only from an enzyme kinetic standpoint but since we must use whole cells the toxicity of this cresol becomes an important consideration that can only be addressed empirically), and duration of test (i.e. time until the rate of TFHA production begins to fall). The effects of oyster shell on the evaluation of TFMP oxidation and protein determination of $PR1_{23}$ were evaluated as well.

(1). pH

A pH range of 6.5 - 10, using a 10 mM Tris-HCl buffer was examined. This buffer was determined previously to be superior in detecting TFHA over similar pH phosphate buffers. The cells used were pelleted from an overnight culture of $PR1_{23}$ grown in LB glucose medium (LBG), and resuspended in the buffer to be tested. The duration of this test was arbitrarily set at 30 minutes. A pH optimum of 8.5 was determined under these conditions (Figure 11).

(2). Oxygen

Various configurations tested provided different surface-to-volume ratios. Theoretically, the optimal expression of Tom requires a maximal oxygen transfer rate. It must be borne in mind that any protocol contemplated must be feasible under field conditions and not require extraordinary steps.

Various vessels were tested, all without agitation (as this would be easiest to accomplish under field conditions) to determine the effect of the surface to volume ratio on the rate of TFHA formation for a single batch culture of one activity.

The original TFMP oxidation tests were performed in 1.5 mL microfuge tubes containing 1 mL TFMP solution. This represents a surface-to-volume ratio of 0.79 cm^2/mL. Despite having a fairly high ratio, this configuration exhibited the lowest TFHA production rate of all vessel configurations tested (Figure 12). The reason remains unclear. All other vessels were glass. It is possible that TFMP is not as available in the plastic microfuge container.

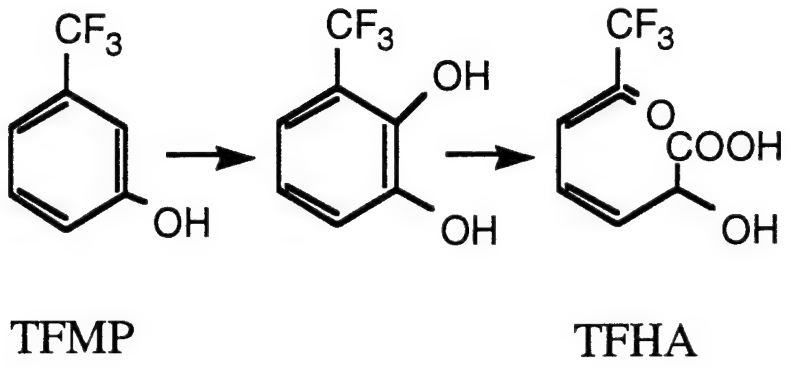


Figure 10. Trifluoromethylphenol Oxidation Pathway

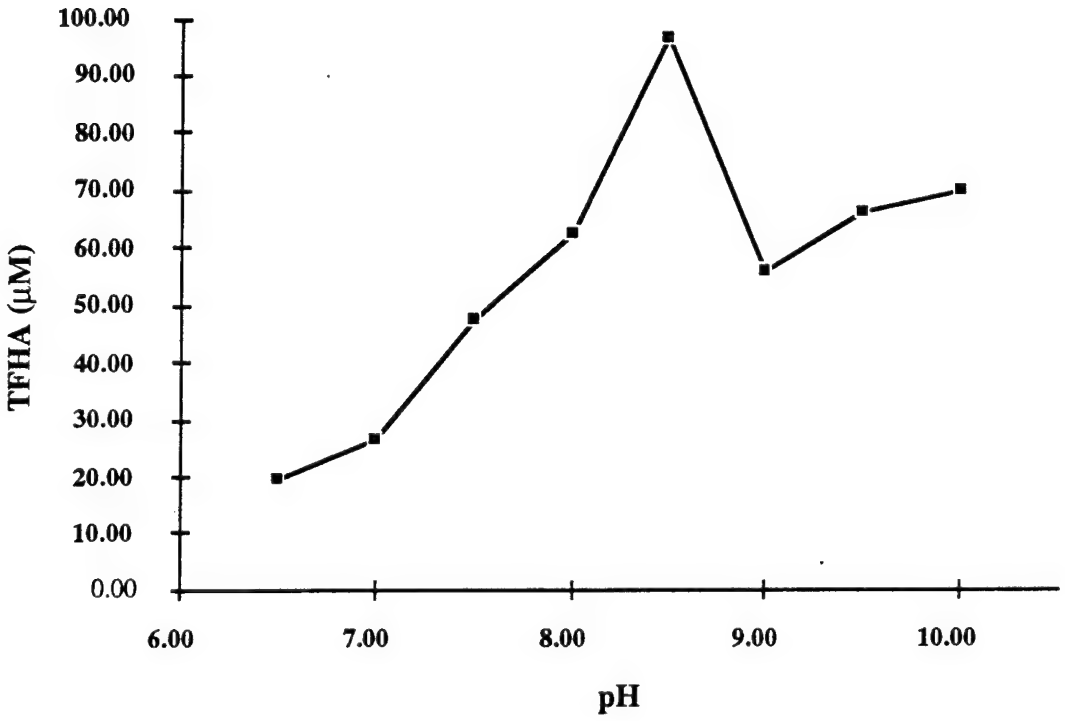


Figure 11. TFHA Production at 30 Minutes

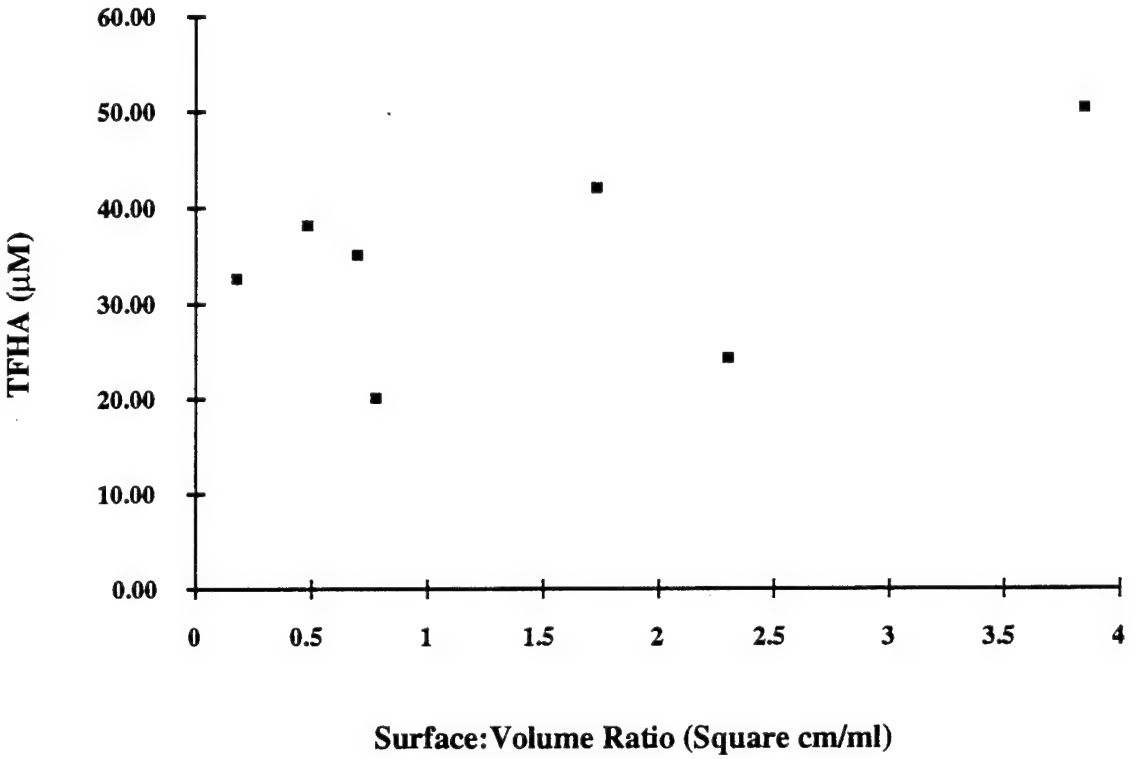


Figure 12. TFHA Production: Surface-to-Volume Effects

The maximal surface to volume ratio investigated (provided by 10 mL of reaction suspension in the bottom of a 250 mL erlenmeyer flask) gave the highest level of TFHA production over a 30-minute period.

(3). Temperature optimum

30°C (see explanation above).

(4). Substrate (TFMP) Concentration

TFMP concentrations of 0.1, 0.5, 1.0, 2.0, and 10.0 mM were tested for 30 minutes Figure 13. The optimal TFMP substrate concentration tested was 1.0 mM. Cresol levels above 1 mM have long been suspected to be toxic to this organism.

(5). Duration of Test and Effect of Support Material

Maximal rates appear to be maintained through 20 minutes. In some of the configurations tested there was a drop in activity after 30 minutes. Since there is ample color production by 20 minutes, there seems to be no need to run the assay longer regardless of the configuration decided upon. In addition the presence of oyster shell was shown not to affect the production of TFHA by active cells over this 20 minute assay period (Figure 14). The same material was also found not found to affect protein determination due to cellular biomass. Only a slight interference by oyster shell was found in the evaluation of $PR1_{23}$ associated protein concentrations by the Pierce Bicinchoninic acid colorimetric method.

To ascribe specific activity to the biofilm it will be necessary to quantitate the biological component. Dry weights are not a useful parameter as the density and irregularity of the oyster shell make this measurement difficult to subtract the abiotic component. Protein analyses offer a solution if they can be used successfully in the presence of oyster shell. Because of the calcium carbonate makeup of the oyster shell, a strong acid buffering effect was feared.

The Pierce bicinchoninic acid colorimetric method for protein determination was tested. As shown in Table 3, there were no observed

effects on BSA protein determinations with or without the oyster shell, or as a result of our 70 °C, NaOH pretreatment. Parallel determinations of cellular protein were derived from different densities of $PR1_{23}$ with and without oyster shell. No differences in protein estimated from $PR1_{23}$ by this method (following a 70 °C, NaOH pretreatment) were seen when oyster shell material was present, nor was there a significant contribution to the overall protein levels from the oyster shell material itself (Figure 15). No impediment to the use of protein assay by the Pierce bicinchoninic acid method to estimate biofilm associated biomass was seen.

Our original tests for TFMP oxidizing activity were conducted in microfuge tubes containing cells suspended in 1 mL (surface to volume ratio of 0.79 cm^2/mL) of 0.5 mM TFMP (10 mM Tris-HCl, pH 7.0), and incubated open (without agitation) for 30 minutes.

As a result of these series of tests, we now perform these evaluations in either 10 or 1 ml cell culture (or 10 or 1 grams oyster shell) in a 250 or 25 ml erlenmeyer flask, respectively. The new buffer contains 1.0 mM TFMP (10 mM Tris-HCl, pH 8.5). Flasks are incubated open (without agitation) for 20 minutes (though our studies indicate that as long as we don't exceed an A_{388} of 3.0 we could easily prolong the test for lower activity material) at 30 °C.

Evaluation of biofilm both in terms of specific Tom activity and protein determination does not seem to represent a particular problem in the presence of oyster shell.

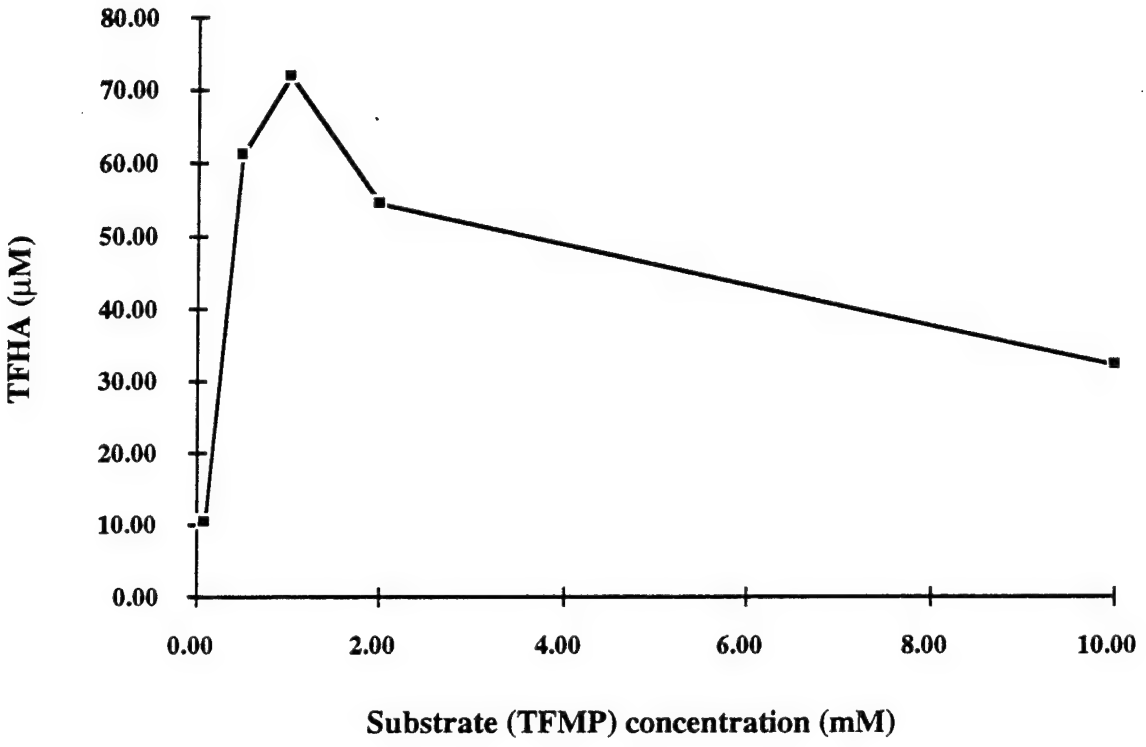


Figure 13. Substrate Concentration Effect on TFHA Production

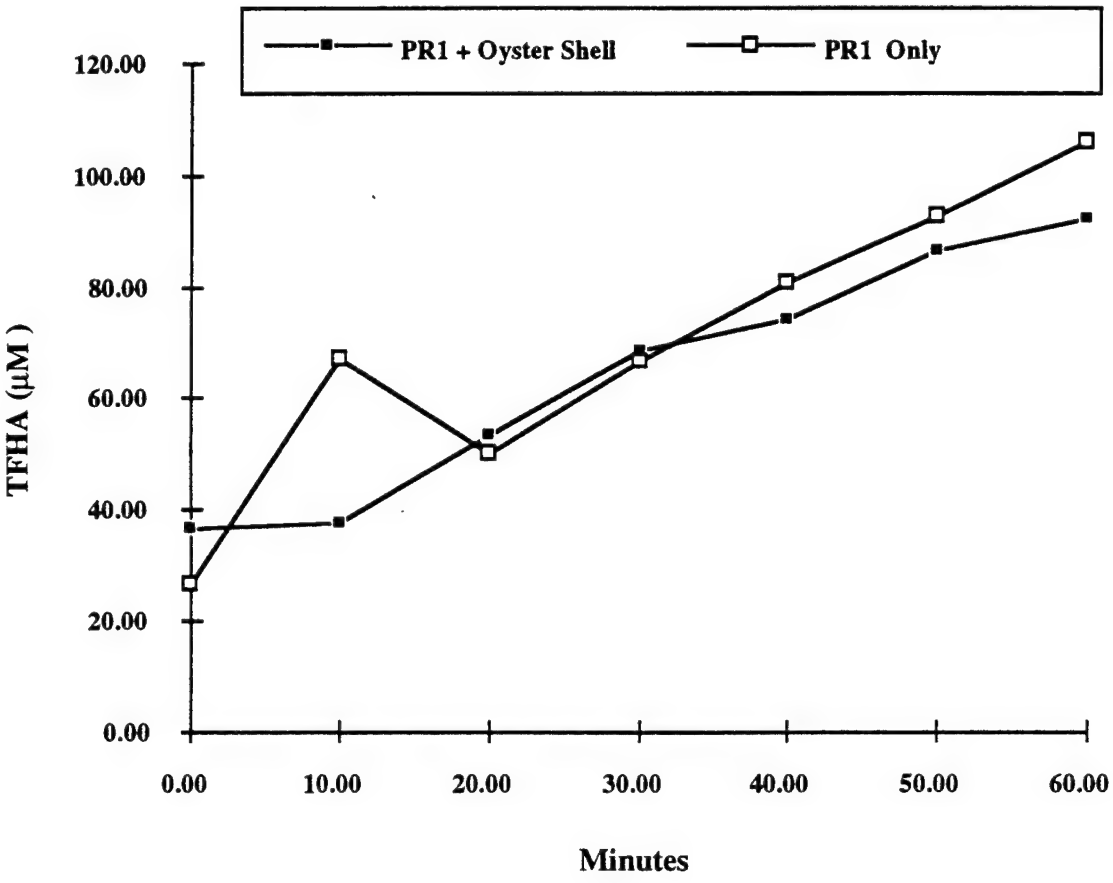


Figure 14. Oyster Shell Effect on TFHA Production by PR123

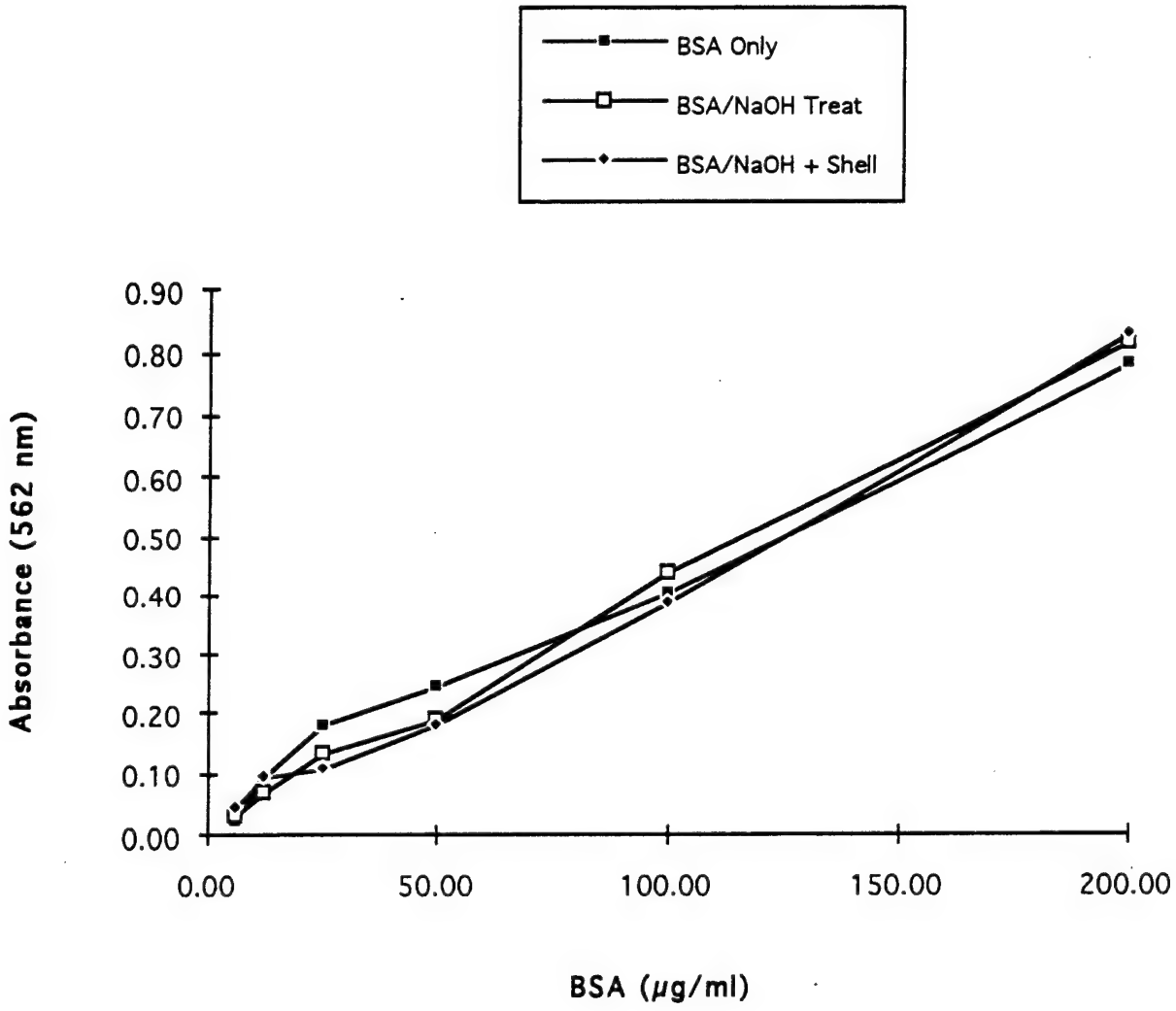


Figure 15. NaOH Pretreatment and Oyster Shell Effects on Protein Determination for Biomass Quantitation

2. Bioreactor Evaluation in the Field

(a). Vapor Phase TCE Treatment.

(1). Reactor Column: Design and Use

Three sample/access ports were installed to accommodate sampling during vapor phase or liquid phase TCE treatment. Conceptual design strategies for the operation of the Tyndall bioreactors included the ability to operate in either an ascending liquid or vapor phase degradation mode.

A schematic representation of the bioreactors as originally used for vapor phase treatment of TCE at Hanscom AFB is given in Figure 16. Only minor modifications of this basic operational design were changed when the columns were switched to liquid phase treatment of TCE.

Two bioreactor schematics detail the flow and recycle patterns used in the vapor (Figure 17) and aqueous phase (Figure 18) experiments for the degradation of TCE. Oyster shell was abandoned in August due to perceived $PR1_{23}$ cultural instability and lack of sufficient colonization (possibly due to excessively high pH buffering capacity of the calcium carbonate material). Two new column support materials were assessed for their ability to harbor $PR1_{23}$. One bioreactor was packed with diatomaceous earth pellets (which had been used successfully in these reactors

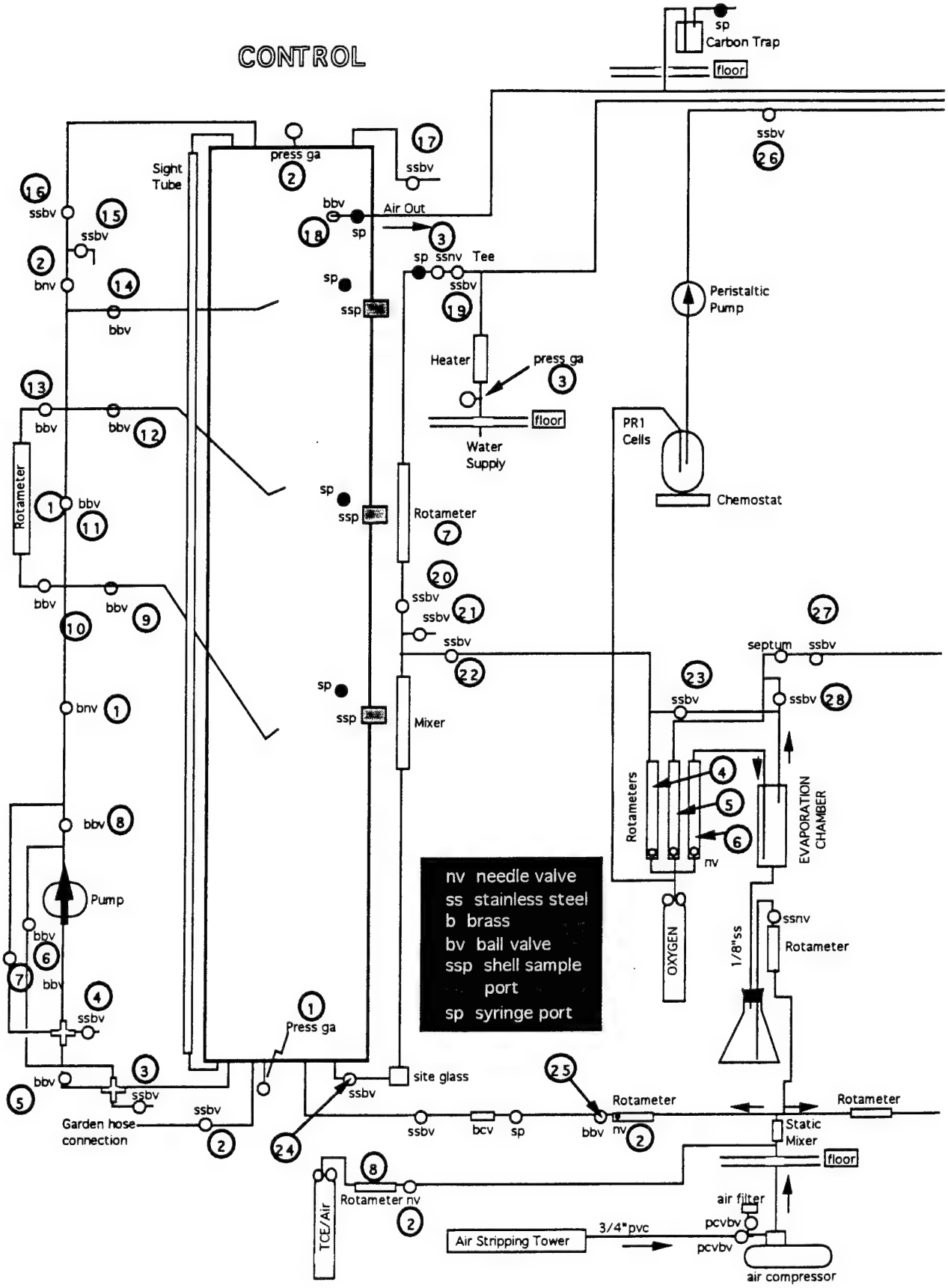


Figure 16A. 100 Liter Stainless Steel Control Bioreactor Diagram

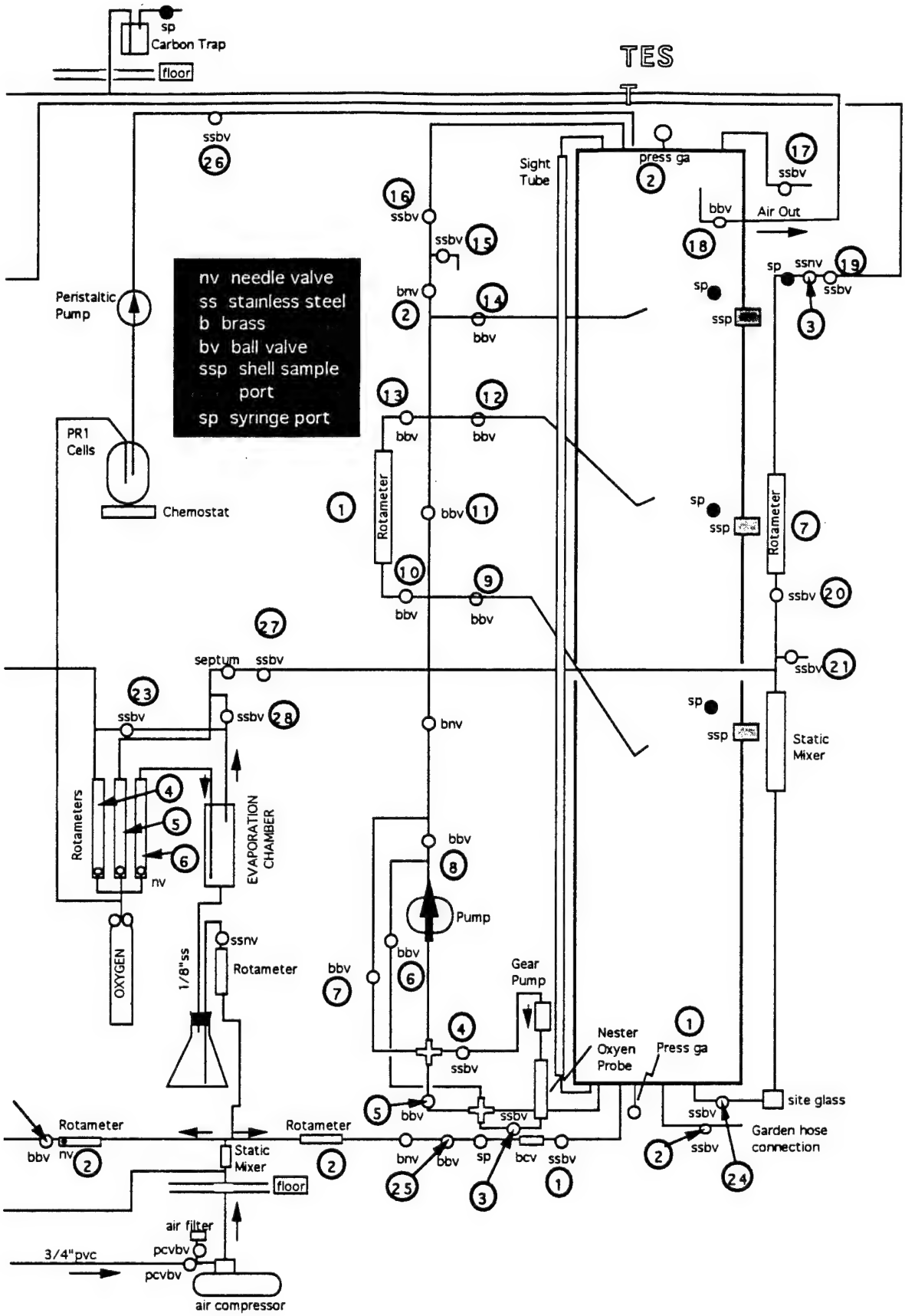


Figure 16B. 100 Liter Stainless Steel Test Bioreactor Diagram

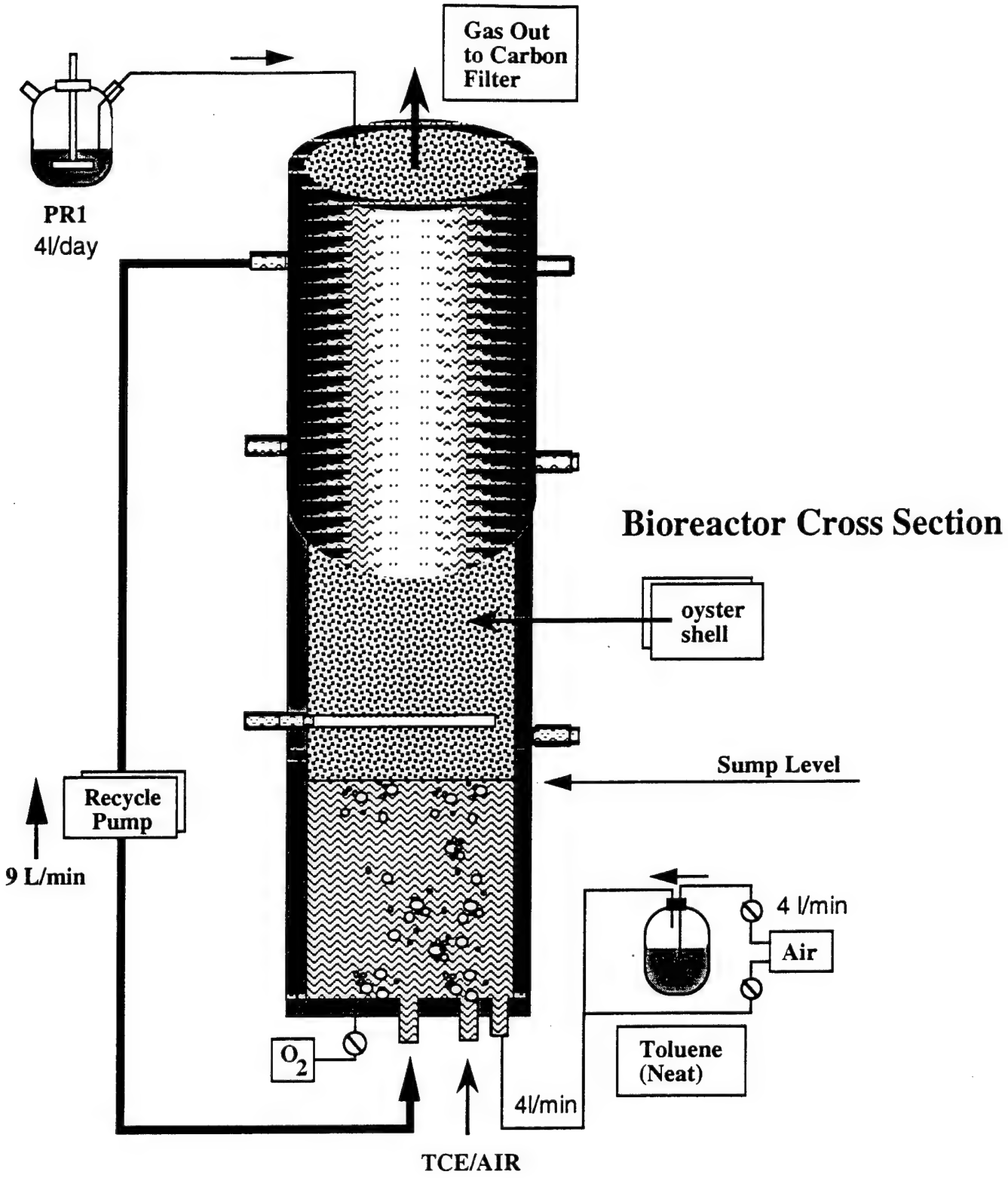


Figure 17. Operation Diagram for 100 Liter Stainless Steel Bioreactor: Vapor Phase TCE Treatment

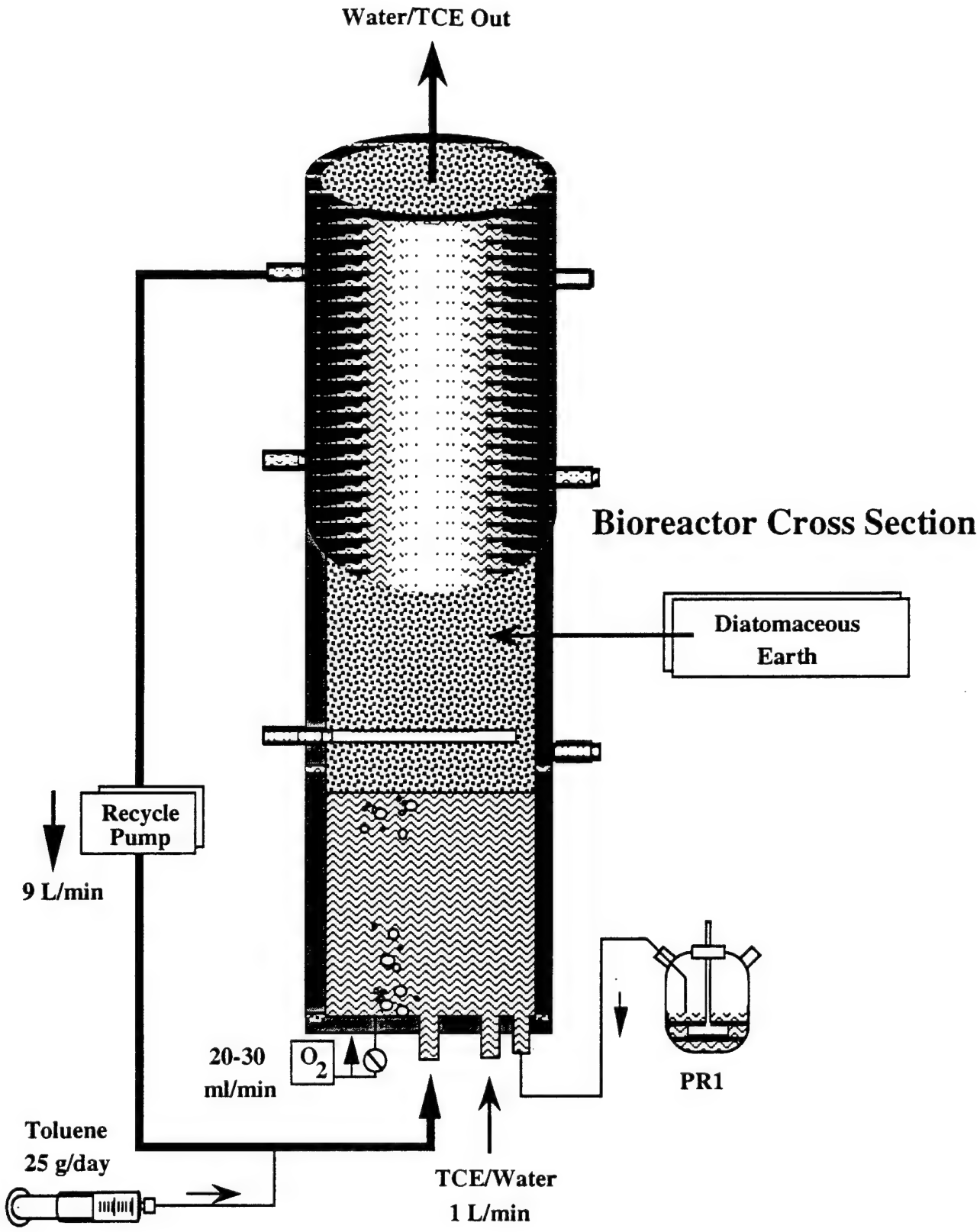


Figure 18. Operation Diagram for 100 Liter Stainless Steel Bioreactor: Liquid Phase TCE Treatment

for the biodegradation of chlorophenols in previous studies by Tyndall personnel) and the other unit with a new ceramic bioreactor material made from clay.

(2). Colonization of the Oyster Shell Bioreactors Under Field Conditions

Effectiveness of colonization was measured in five ways:

(a). TFMP oxidation rates from column packing samples.

(b). Protein measurements from column packing samples.

(c). Bacterial counts of material resuspended from column packing samples or sump liquid.

(d). TCE degradation rates of column packing samples based on headspace analysis in bottles.

(e). Changes in input hydrocarbon concentrations throughout the column.

Despite these preliminary experiments which suggested the utility of oyster shell as a support matrix for $PR1_{23}$, results from the field trials suggested that other previously unconsidered factors prevented successful colonization. Oyster shell was colonized for only a short distance (the upper few centimeters of the column) past the input source of buffer and nutrient (toluene). This zone of growth persisted for several weeks, but gradually disappeared. Subsequent laboratory studies indicated that the pH of the liquid within the oyster shell column was in fact inhibitory to $PR1_{23}$ growth and colonization. The initial decision to use this method for colonization stemmed from (what now must be viewed as a misinterpretation) of laboratory scale studies with the same buffer system and carbon source in which much shorter columns resulted in extensive colonization and activity. Presumably the microenvironments of these smaller columns were more successfully buffered with the relatively strong phosphate buffering capacity of BSM.

Toluene fed at an air concentration of $\sim$ 5 mg/liter of air was previously determined in the laboratory to be an efficacious route of carbon addition for the establishment of a heavy biofilm of $PR1_{23}$ (i.e. >1 mg cellular

protein per gram wet oyster shell) with high specific activity. Toluene fed as the sole carbon source in this manner was adopted as the most desirable route for colonization of the reactors under field conditions at Hanscom.

Two reactors were used. One received $PR1_{23}$ inoculum, basal salts medium, toluene, and air, and was termed the test reactor. The other received the same treatment without $PR1_{23}$, and was the control reactor.

$PR1_{23}$ was grown continuously in a phthalate-fed chemostat at a dilution rate of ~ 2 day^{-1}, and pumped onto the top of the test reactor bed for the first 10 days. Meanwhile recirculation of a 20-liter sump of 1x basal salts medium (BSM) at ~ 9 liters/minute was carried out over the entire column. This recycle rate was reduced to ~ 200 ml/ min after the 5th day. Toluene vapor was introduced via an independent air stream (~400 ml/min) to the bottom of the sump apart from the main air stream (4 L/min) also entering at the sump bottom. The final concentration of toluene in the resident air of the reactor was calculated, based on the total input of toluene and the total volume of air from both sources per unit time.

(3). Physical Monitoring of Reactors

The reactors were daily monitored for temperature, pH, liquid recycle and gas flow rates (i.e. air (with or without TCE), air with toluene, and pure oxygen). The concentration of toluene within the air/toluene pulse was calculated from the air flow rates and the rate of toluene disappearance from the feeder flask (monitored daily by change in mass). The sump level, pressure differential between the top and sump of the reactor (none seen), and the oxygen concentration of the recycled sump liquid were also monitored.

The concentration of toluene in the air /toluene pulse, as well as its duration and feed rate were the same for both the control and test columns. These are presented in Figure 19. Toluene was fed to the reactors at a rate of approximately 12 L/hr for the first two weeks, and then increased to ~ 28 L/hr for the next two weeks. The toluene concentration during these two periods remained largely between 4 and 10 mg/L air. On July 20, pulsing began with two periods of toluene feed at approximately 28 L/hr for 2 hrs each, once in the morning and once in the afternoon. During this time it was noted that the

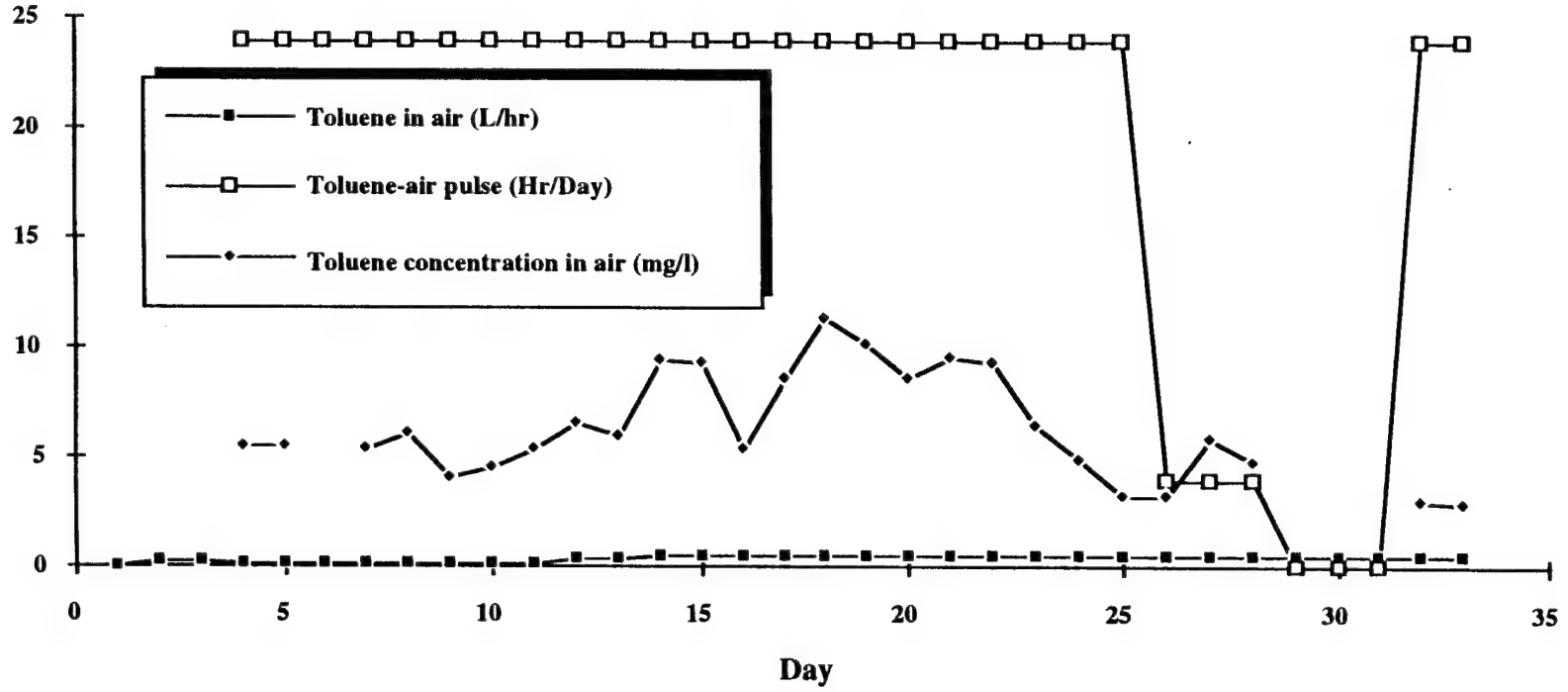


Figure 19. Toluene Application Schedule to Vapor Phase Bioreactors

biomass of the test column was not as high as it might be. Since the toxicity level of toluene to $PR1_{23}$ is still a matter for debate (in regard to a fixed biofilm on oyster shell) it was decided to try increase the number and mass of active cells through the addition of an alternate carbon source; Phenol, which is utilized via the same pathway, and for which the toxicity was well established (> 5μM). Approximately 30 liters of 2 mM phenol in 0.5 x BSM was pumped onto the column over the weekend of July 23 and 24 (see arrow Figure 20). Toluene readdition was begun with a pulse duration of 24 hours on July 25. Phenol input was stopped at the same time.

The temperature, pH, air input rate and oxygen concentration of each columns recycle line are presented for both the test and control reactors in Figure 20. Though there were no differences in air input rates or temperatures for the two there were some interesting deviations in both pH and oxygen content of the sump liquid. A substantial, and sustained pH difference was noted between the two columns. Also note the degree of oxygen depletion in the sump liquid of the test column, relative to the control column following phenol addition Figure 21.

(4). Chemistry and Biochemistry

(a). TCE and *cis*-DCE in/output

TCE degradative potential of the oyster shell column was monitored following 41 days of colonization. TCE degradation was determined from air routed from the air stripper at the Hanscom facility through the test and control columns, from August 4 through 12 at a rate of 2 L/min.

Figure 22 indicates the TCE mass balance for the abiotic control and the test columns. No TCE loss was detected at any point within the column. The oyster shell packing did not evidence any perceptible sorption of the TCE as indicated by the rapid recovery of the full influent TCE concentration in the effluent gas at the first sample point. These control results were then used to compare to those of the test (or biotic) column. TCE levels were again the same at all points tested. While there was a small degree of TCE removal in the test column relative to the control it must be considered insignificant.

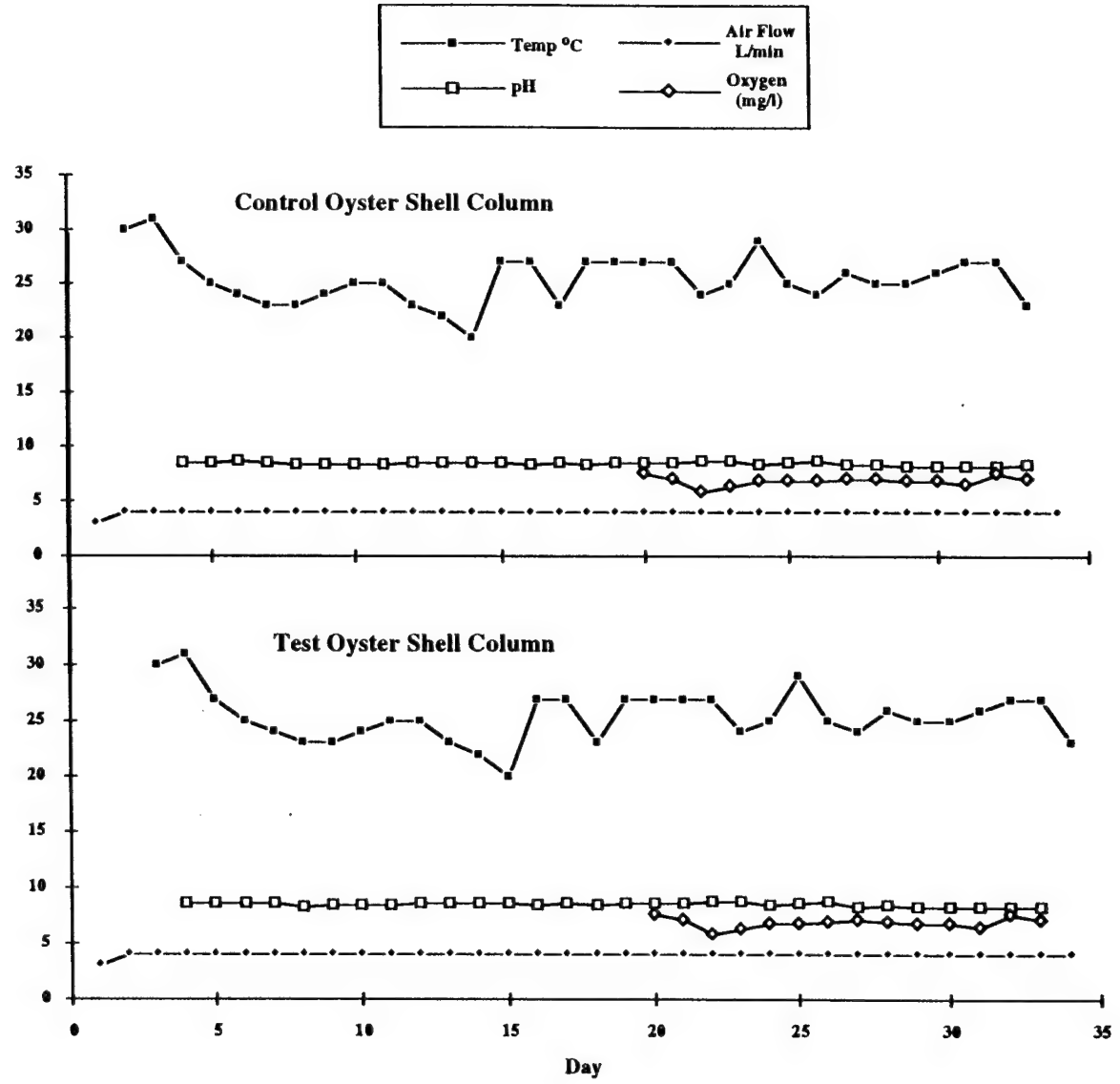


Figure 20. Physical Parameters of the Vapor Phase Columns During Colonization

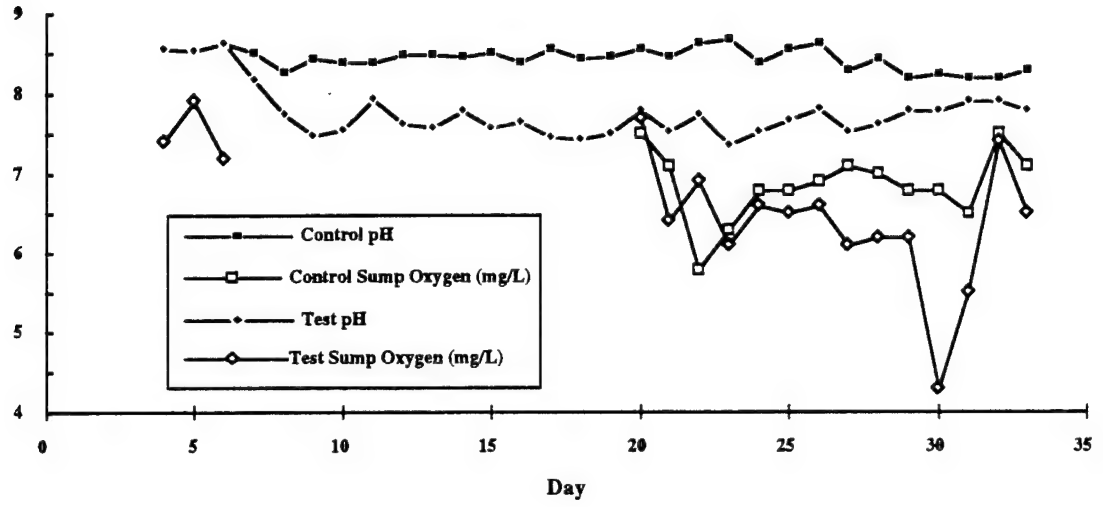


Figure 21. Comparison of Oxygen and pH of the Test and Control Vapor-Phase Bioreactors

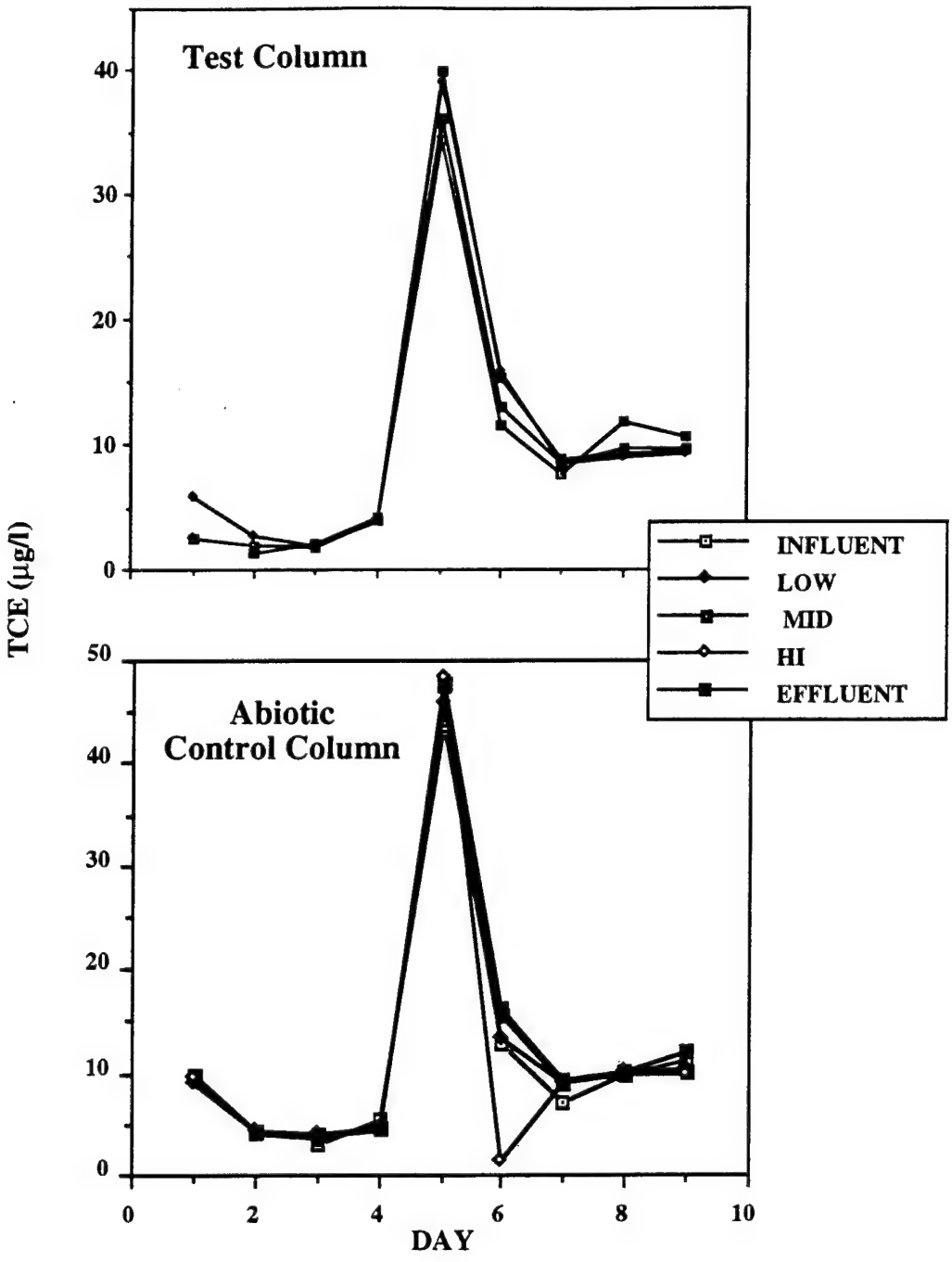


Figure 22. TCE in the Input and Effluent Air Streams of the Test and Control Bioreactors

(b). Tom activity

To establish the degree of colonization of the oyster shell it is necessary to measure the biofilm directly (through protein determinations) and indirectly (through Tom activity towards TFMP). The first was done through protein measurements taken from column material at various levels twice weekly. The oyster shell itself gives a background protein concentration of ~ 50-100 μg protein/gram wet weight oyster shell. The results of horizontal core samples analyzed for protein content are presented in Figure 23. Protein yields are presented per gram wet weight oyster shell (corrected for the detectable protein content of the oyster shell). In addition to biomass the activity of the biomass to the cresol analog TFMP was determined from the same samples as used for the protein determinations above. In this way the total activity in terms of TFHA produced per unit time by the oyster shell material from the two columns could also be related to its specific activity.

The data related in Figure 24 from samples taken at the Hi, Mid and Low ports of the test column, clearly indicate the highest Tom activity is from the same samples yielding the highest protein determinations. The control column was sampled at the Mid port only. These demonstrated only background readings (<0.2 nmoles TFHA/min/g oyster shell). Samples from the control column never showed any change in protein content beyond the background normally measured for the oyster shell alone. TFMP oxidative activity in the test column was easily demonstrated in the High region of the test column that initially received $PR1_{23}$ and toluene, for a ten day span (beginning on day 16 of operation). Unfortunately, this activity had already decreased to background levels by the time vapor-phase TCE was introduced. We were unable to maintain $PR1_{23}$ as an active and significant member of the oyster shell community under these conditions.

An overlay of TFMP oxidation rate and protein content for the high port of the test column is given in Figure 25. The resulting Tom specific activity toward TFMP (given as nmoles TFMP oxidized per minute per mg protein) at this high port of the test column is presented in Figure 26. Tom activities in nmoles TFHA/min/gram wet weight oyster shell) for all levels within the test column are given in Figure 27.

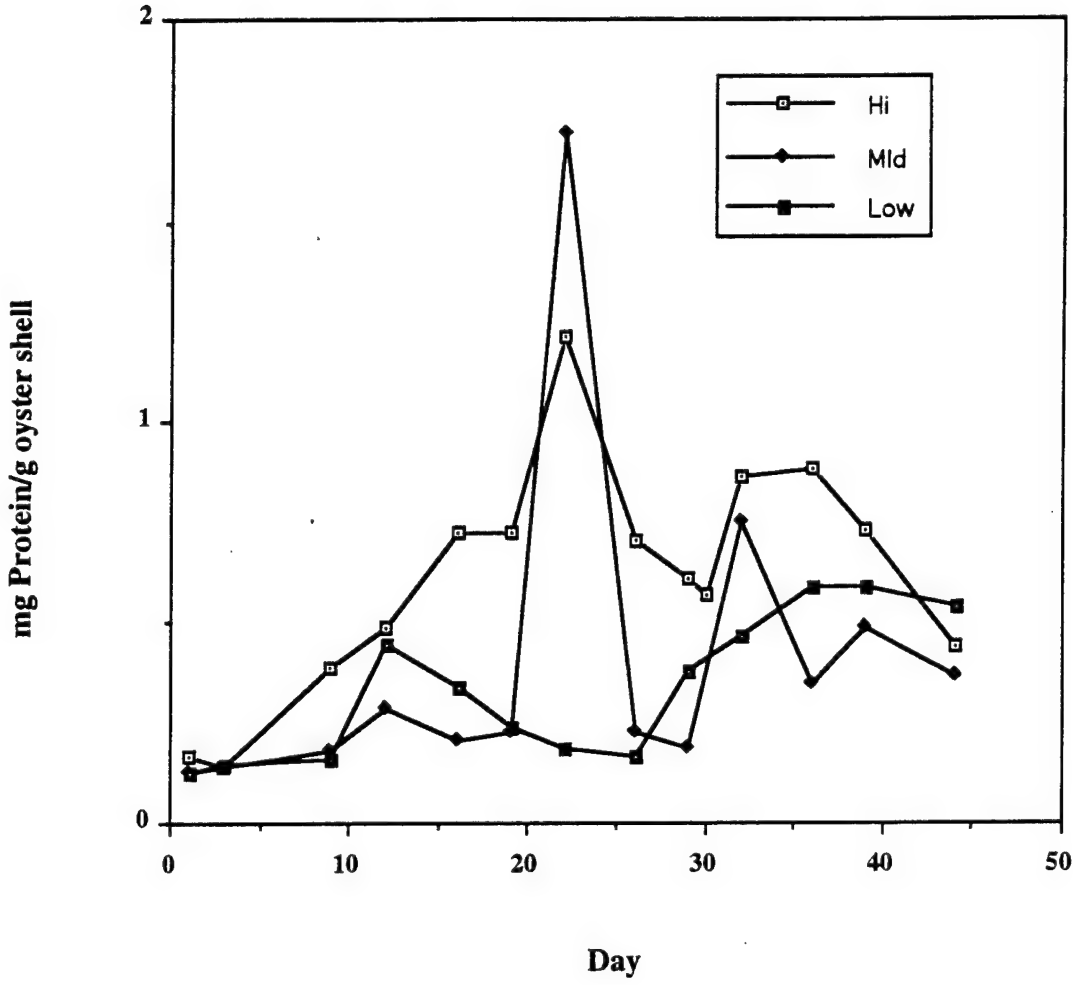


Figure 23. Biomass Estimations from the Test Column Oyster Shell Packing

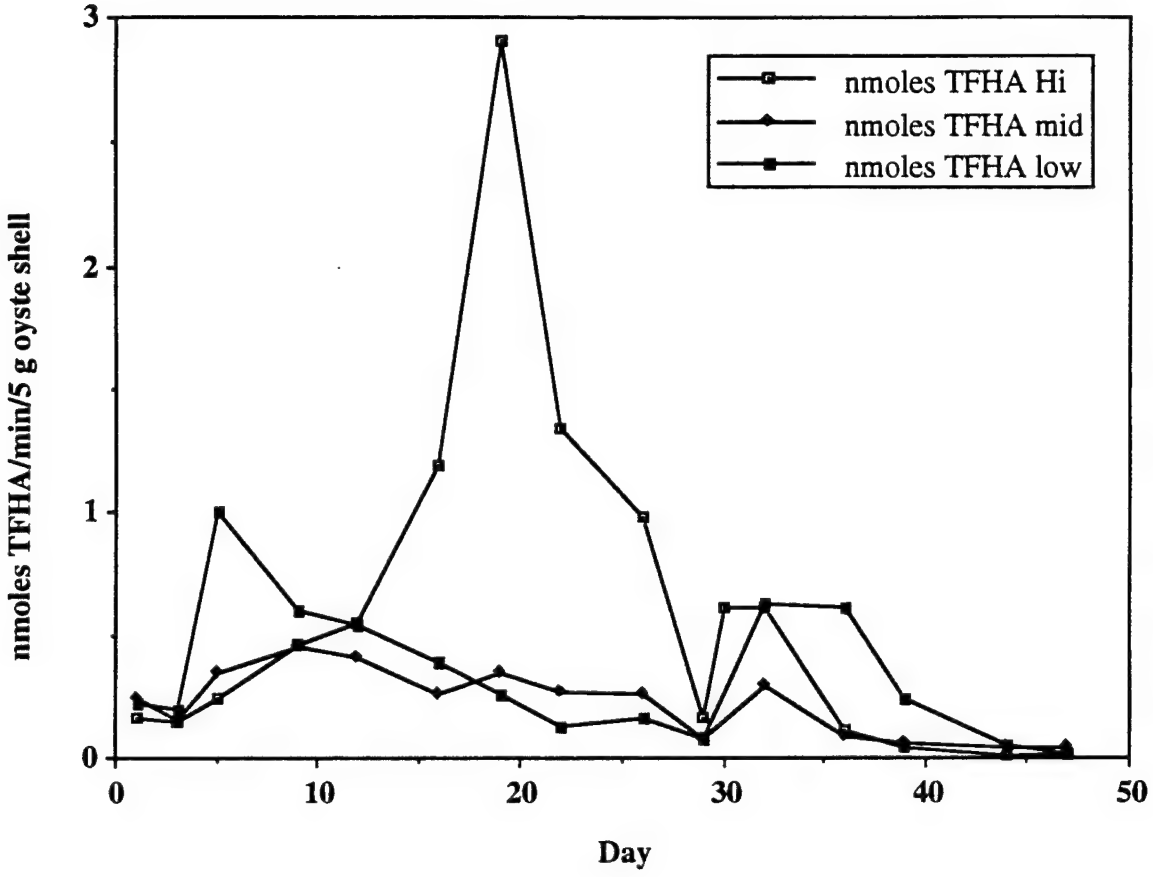


Figure 24. TFHA Activity of the Oyster Shell Packing of the $PR1_{23}$ Inoculated Column

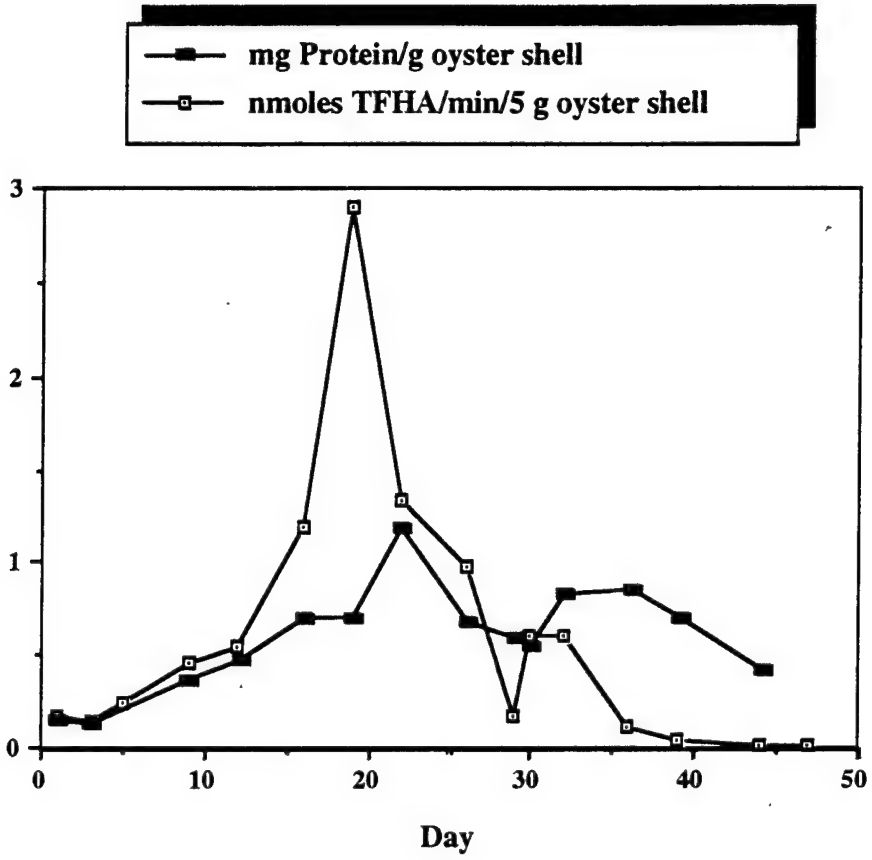


Figure 25. Biomass and Tom Activity Relationship in Top Port of $PR1_{23}$ Amended Reactor

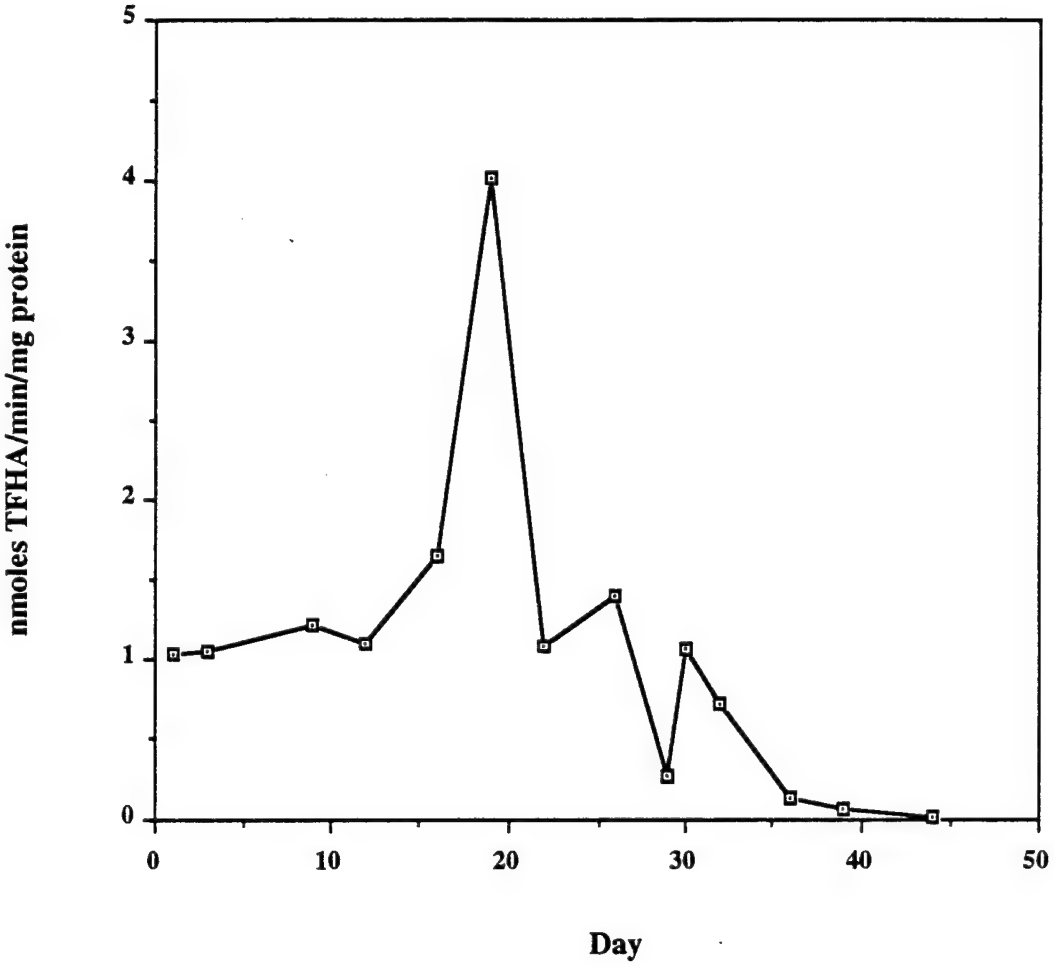


Figure 26. Tom Specific Activity, High Port $PR1_{23}$ Test Column

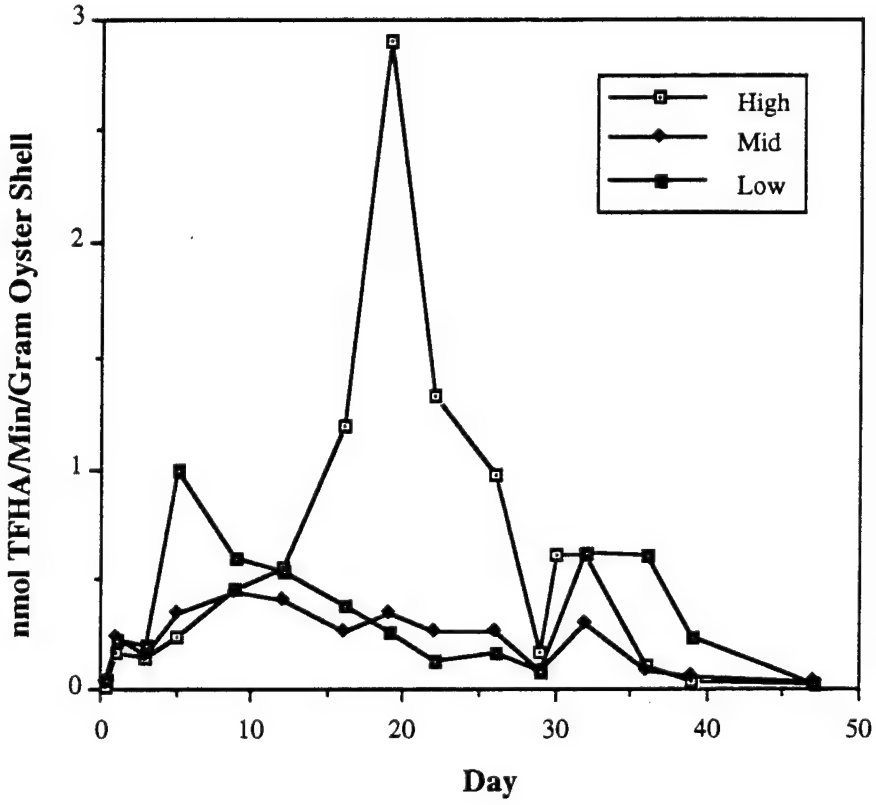


Figure 27. TFMP Oxidative Activity Throughout the $PR1_{23}$ Test Column

This could be due to a variety of reasons. One is that the inoculum did not filter to lower regions of the column adequately and therefore only the very top was successfully colonized. If this is the case, one would presume the lower regions would colonize if given more time, providing a large excess of $PR1_{23}$ cells would not be required to establish themselves in the face of competition. Another, perhaps more tenable hypothesis, is that the toluene input concentration is simply too high. The actual concentration of the toluene input air stream is ~46 mg/L air as it bubbles into the sump liquid. As it equilibrates with the other air source it is diluted to ~5 mg/L air throughout the rest of the column. Despite laboratory measurements of 5 mg toluene/L air as a concentration sufficient to give good colonization of oyster shell. A very high concentration of toluene may exist at the bottom of the reactor, and this exceeds the toxic threshold of $PR1_{23}$. This would explain the virtual absence of biomass or activity from the sump liquid, and the progressively better colonization as one moves up the column.

The toluene input concentration was reduced from ~ 5 mg/L air to ~3 mg/L air on July 26, and further reduced on July 30 to try to achieve a target concentration of 0.5 mg/L air.

(c). Microbiological monitoring of the vapor phase reactors

The microbiological assays developed for monitoring $PR1_{23}$ populations in the biofilm did not perform as well as we had hoped. The single biggest problem is an inability to develop a reproducible assay for TFMP oxidation by individual colonies. It was hoped that this assay could have been employed to determine the presence of $PR1_{23}$ on noninducing media like LBG.

Total heterotrophs were found to increase approximately three orders of magnitude within the first weeks throughout all levels of the test reactor, and then level off at somewhat reduced population densities (~10^9 cells /gram of oyster shell vortexed with 1 ml of BSM for 30 s) Figure 28. Kanamycin resistant heterotrophs (to which $PR1_{23}$ would belong) also enumerated from these samples are shown in Figure 29. For these cells there were no clear trends apparent during the first 2 weeks, that were so evident

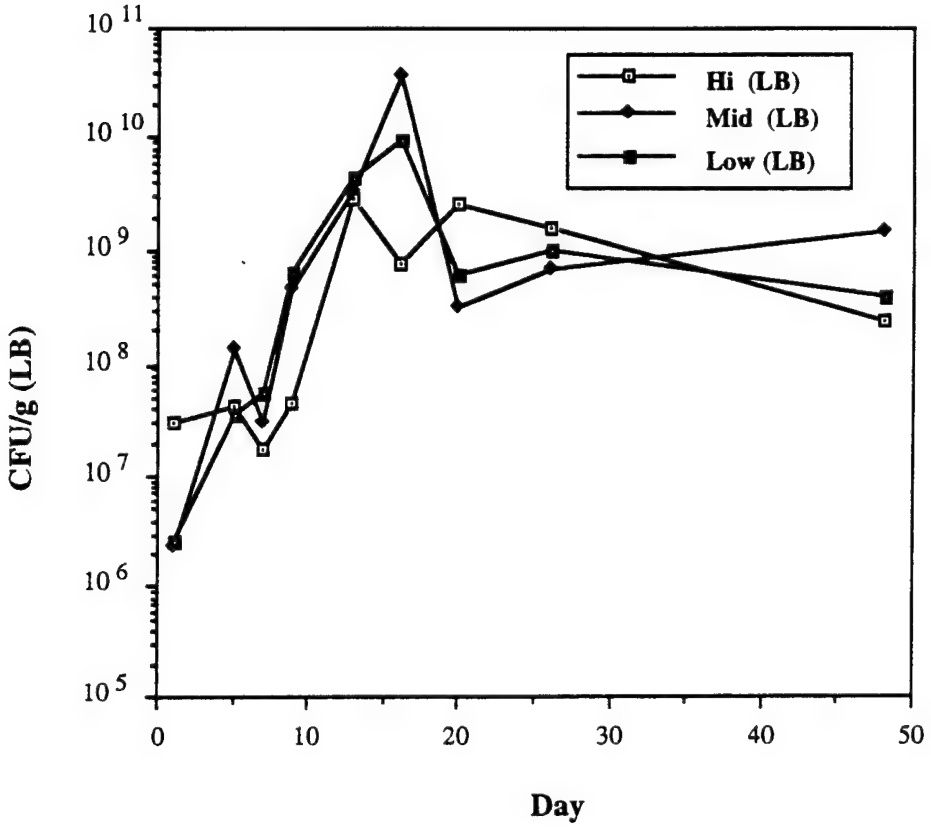


Figure 28. Heterotrophic Bacterial Populations Throughout the $PR1_{23}$ Test Column

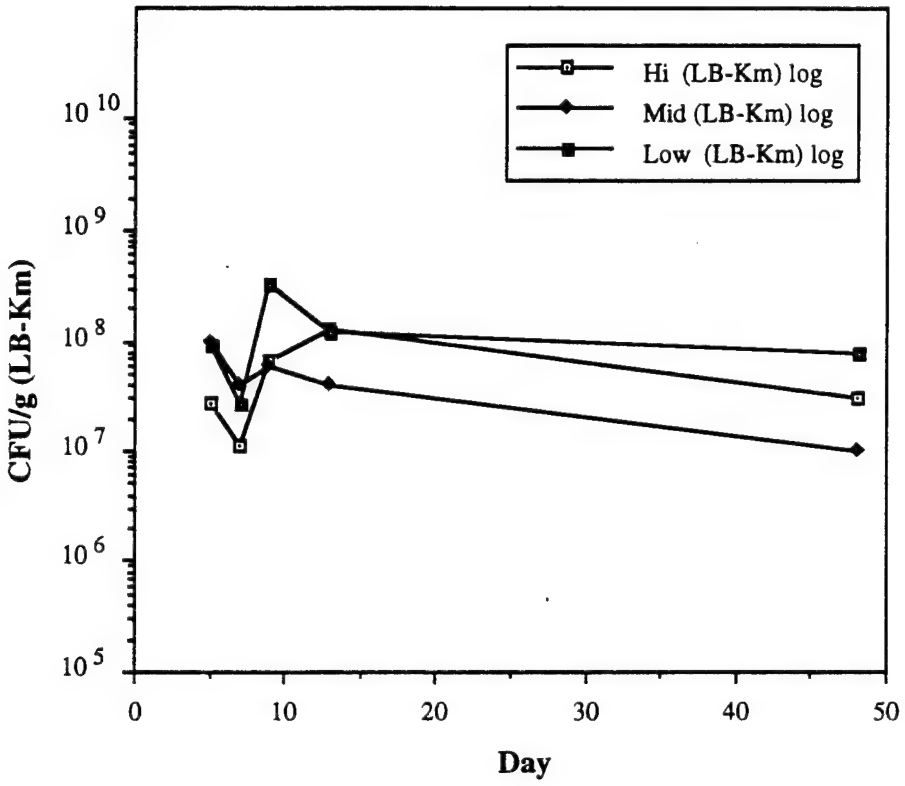


Figure 29. Kanamycin -Resistant Heterotrophic Bacterial Populations Throughout the $PR1_{23}$ Test Column

for the total heterotrophs, and they seemed to stabilize at a few percent of the total heterotrophs. This would seem to be evidence for shell colonization by bacteria other than $PR1_{23}$ (assuming that kanamycin resistant growth by $PR1_{23}$ was not merely selective of nondamaged or more healthy representatives). One might argue there was a discernable increase in the Km-resistant numbers in Hi samples during this period (~ one log increase). This would only account for ~10-20% of the total heterotrophs over this same period. Enumeration of phenol utilizers (Figure 30) revealed an approximate 100-fold increase in the levels of these bacteria over the first 20 days. Again these heterotrophic and phenol-utilizing populations appear to mirror the behavior seen in biomass and Tom activity measurements.

(d). Reactor physical parameters

Temperature and pH values recorded during the vapor-phase degradation study are presented in Figure 31. The pH of the inoculated column was consistently lower than that of the control column, despite the same treatment otherwise. The uninoculated oyster shell maintained a pH between 8 and 8.5 for the duration of the study.

Previous experiments indicated the Tom enzyme was active over a pH range of 5 - 9, however no studies had been performed on pH ranges affecting growth. Therefore, laboratory experiments were conducted to ascertain the range of pH in which $PR1_{23}$ would grow. 20 mM Phthalate supported growth between pH 6 and 7.5. Following growth at these pH's, the cultures were analyzed and the final pH determined to be between 8.0 & 8.5. Upon acidification to pH 7.0 they yielded substantially more growth. It was concluded that this upper pH range 8-8.5 represented a barrier to the successful growth of $PR1_{23}$ on such oyster shell in the field. Presumably the smaller columns used in the laboratory were more significantly buffered, therefore allowing a greater growth yield of $PR1_{23}$ on oyster shell.

Air-phase TCE (from the Hanscom strippers) was routed to the reactors on July 20, and continued for nine days. During this time the TCE concentration of the influent gas varied from 390 to 1,050 ppb (µg/l). no

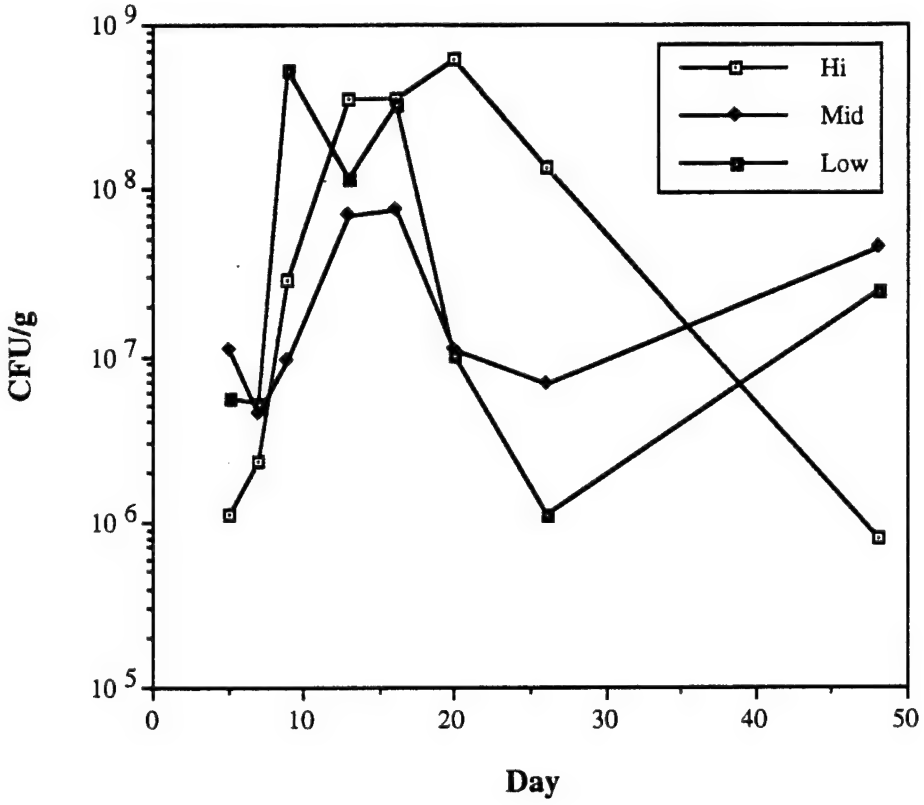


Figure 30. Phenol -Utilizing Bacterial Populations Throughout the $PR1_{23}$ Test Column

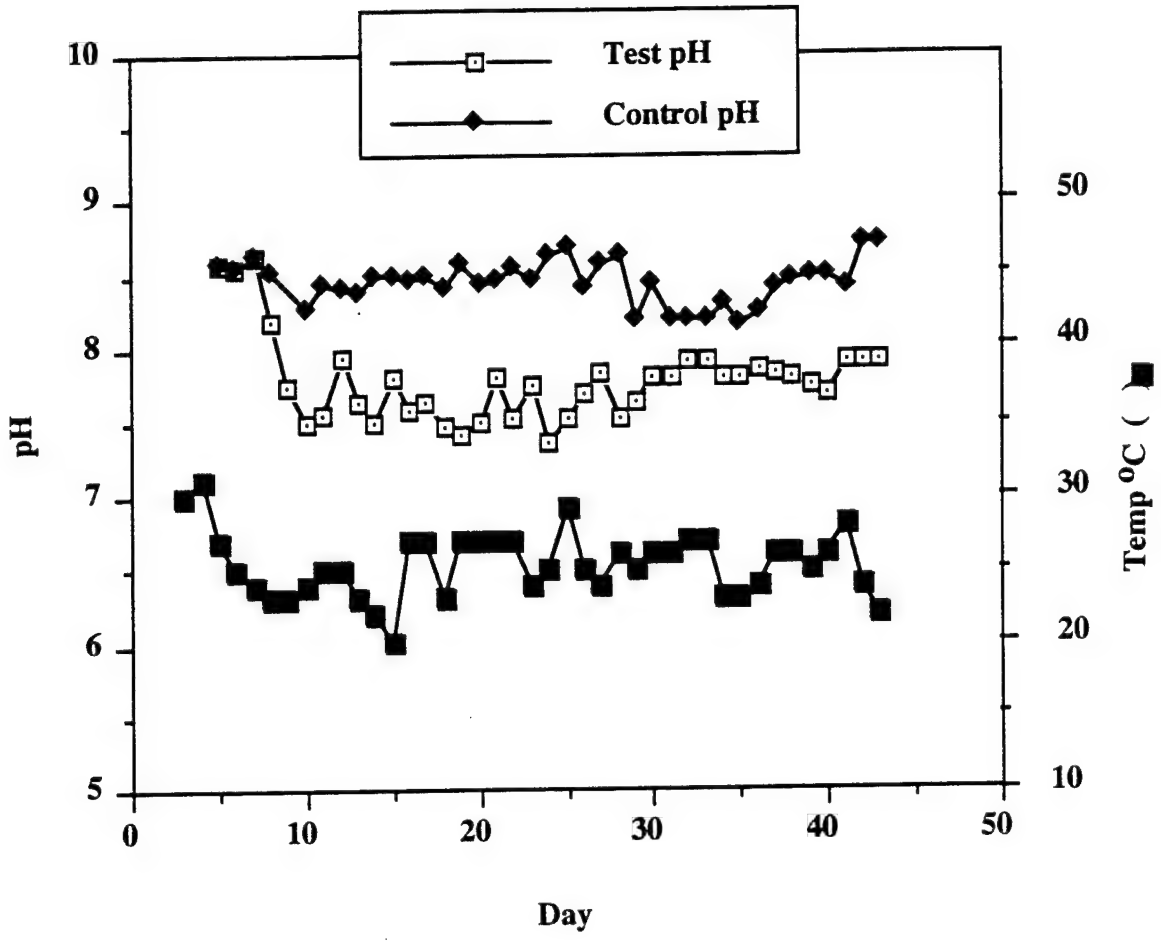


Figure 31. Temperature and pH of Test and Control Columns

significant differences between the colonized and control reactors were seen for the degradation of vapor phase TCE (Figure 22). This is not surprising since all detectable activity was lost prior to vapor phase TCE exposure.

3. Aqueous Phase Treatment

(a). Reactor Column: Design and Use

Contaminated water from the Hanscom site was diverted to the bioreactors prior to air stripping. The contaminated water was then passed through the reactors into a hypochlorite treatment chamber where escaping $PR1_{23}$ were subjected to 0.05% sodium hypochlorite, and returned to the liquid equilibration tank which provides the feed material for the air strippers. The flow pattern of the liquid-phase reactor is given in Figure 18. Because of the failure of the oyster shell in the vapor-phase of the field test it was decided to try two alternative bioreactor support packing materials: pelleted diatomaceous earth (produced by the Manville Corp.) and "type-Z" ceramic pellets manufactured by Grace Sierra. The control column was repacked with the type-Z pellets and the test column with the diatomaceous earth pellets.

Contaminated water was routed through each column at ~ 1 l/min, giving a complete column volume turnover time of ~ 65 minutes, or ~ 22 column volumes per day (at void volume of ~ 65 l). Cells were added each morning after that days TCE samples had been taken. This meant that ~ 22 column volume dilutions of the freshly added cells had taken place prior to a measurement of TCE degradation efficiency. It was felt that this experimental design would not bias the results because of cells in suspension, and would in fact represent the activity of cells somehow "attached" to the column matrix. An experiment was run on day fourteen (the day before the last cell addition) to determine whether the degradative activity was associated with the recycle liquid or the pellet material in the diatomaceous earth pellet column.

During the aqueous-phase treatment of TCE, it was decided to recycle the columns from the mid to bottom while all inputs were from the bottom and all outputs from the top. This design was intended to test the hypothesis that the lower levels of the reactor could be used essentially as a continuously

stirred tank reactor, while the upper half would exhibit characteristics of a plugged flow reactor. Interestingly the diatomaceous earth reactor (at least) seemed to behave as a plugged flow reactor over its entire length with respect to toluene degradation, but as a fully mixed reactor with respect to TCE and *cis*-DCE.

(b). Colonization of the Oyster Shell Bioreactors Under Field Conditions

Inoculation of these reactors was by daily input of overnight cultures raised on 20 mM phthalate, phenol, or phenol overnight followed by LB addition for an additional 24 hours. The rationale was to provide the column with an overwhelming inoculum, even in the face of certain contamination. The plan was to provide the column with a continuous source of somewhat selective carbon source. In this case it was to be toluene supplied in the neat form via a syringe pump at the rate of 25-30 grams per day. It was decided that the most efficacious way to generate $PR1_{23}$ biomass was to supply toluene at the maximal rate the reactor would completely utilize it, i.e. where effluent levels were non-detectable or very low.

(c). Physical Monitoring of Reactors

This was performed as described above for the vapor phase portion of this experiment.

(d). Chemistry and Biochemistry

(1). TCE and *cis*-DCE in/output

TCE and *cis*-DCE input concentrations ranged from an initial high of ~550 µg/l to a low of ~220 µg/l, over the course of this study. Figure 32 indicates the concentration of TCE at the Influent, Sump, Low, Mid, High and Effluent sample ports of the diatomaceous earth reactor. It is readily apparent that the loss of TCE was equal throughout the column. This would indicate that with respect to TCE the column operating at a recycle of 9.5 L/min in the Mid to Sump route behaved as a continuously mixed batch reactor. *cis*-DCE was found to behave in a like manner (with all levels

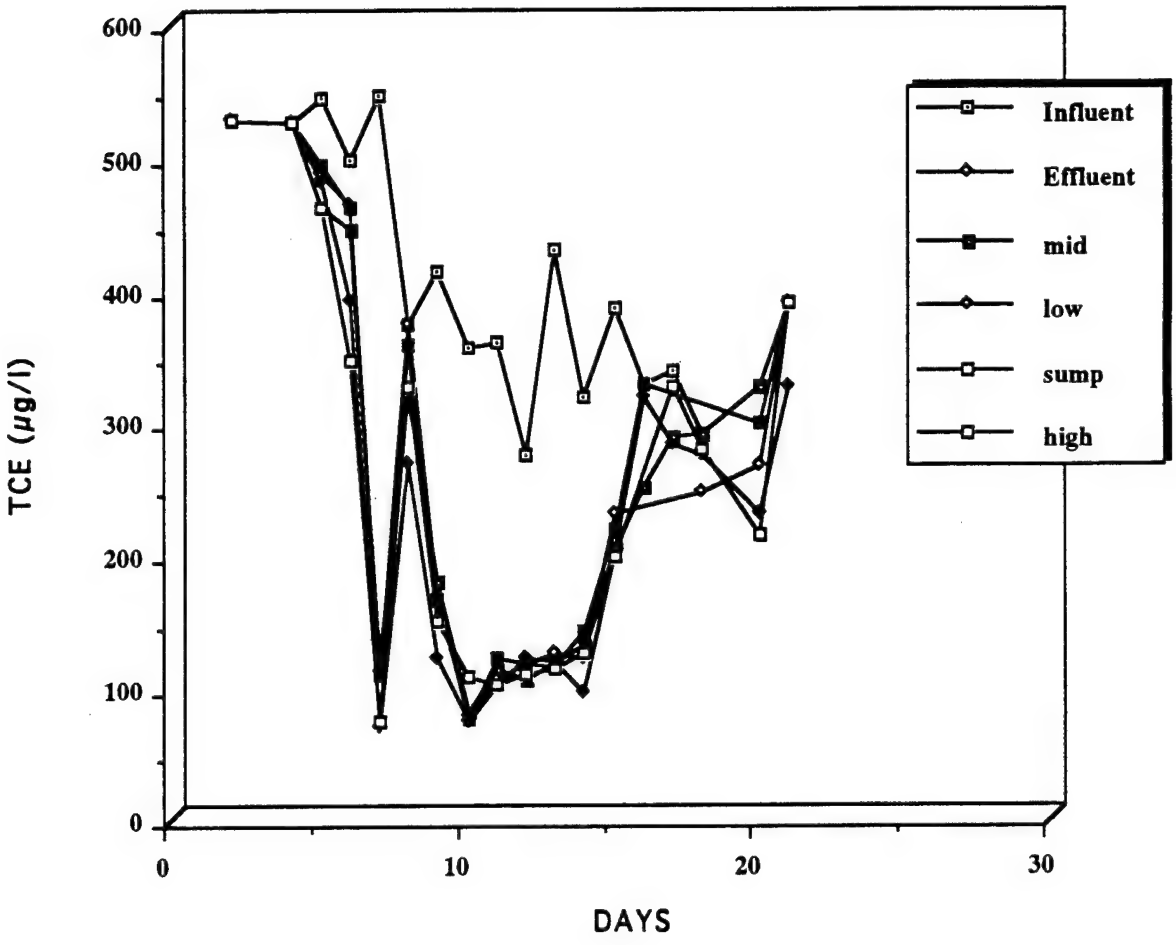


Figure 32. Aqueous-Phase TCE Degradation at All Levels in the Diatomaceous Earth Column Inoculated with $PR1_{23}$

yielding the same concentration). The influent and effluent concentrations of *cis*-DCE and TCE are presented for the diatomaceous earth pellet column in Figure 33. The relatively greater capacity for rapid removal of *cis*-DCE is evident. The sudden loss of degradative activity on Day 15 coincided with the introduction of ~40 mL of neat toluene into the column. By inhibiting TCE and *cis*-DCE degradative activity, it serves as an excellent control in that the biological functions were selectively inhibited, resulting in an immediate return to an effective mass balance recovery of the two chloroaliphatics in the effluent waste water.

The superior performance of the diatomaceous earth pellets in the performance of the bioreactor is clearly seen in comparison with the Z-pellet reactor (Figure 34). Here the levels of TCE at all levels of the reactor nearly matched that of the input water supply, only transiently indicating what might be degradative activity toward the end of the experiment between days 12 and 17.

(2) TFHA activity

While TFHA activity was an excellent measure of colonization of the oyster shell, it was never detected in samples of either pellet type. The pellets themselves were shown not to affect the reaction so it must be concluded that despite the TCE degradative activity the density of active cells was below the limits of detection of this particular assay. This may point to a homogeneous distribution of active cells throughout the column, but at a very low density (measured at ~10^9 per gram in the microbiological assays noted below).

(3). Toluene concentration/output

There were only traces of toluene detected in the site water (<10 µg/L). Daily additions of 25 - 30 grams of neat toluene to the reactor sump via the recycle line were made to feed the column inocula over the 24 hour period (i.e. at 1.03 - 1.25 g/hr). This was calculated to give 17,000 - 21,000 µg/L toluene in the effluent had no degradation occurred. The concentrations

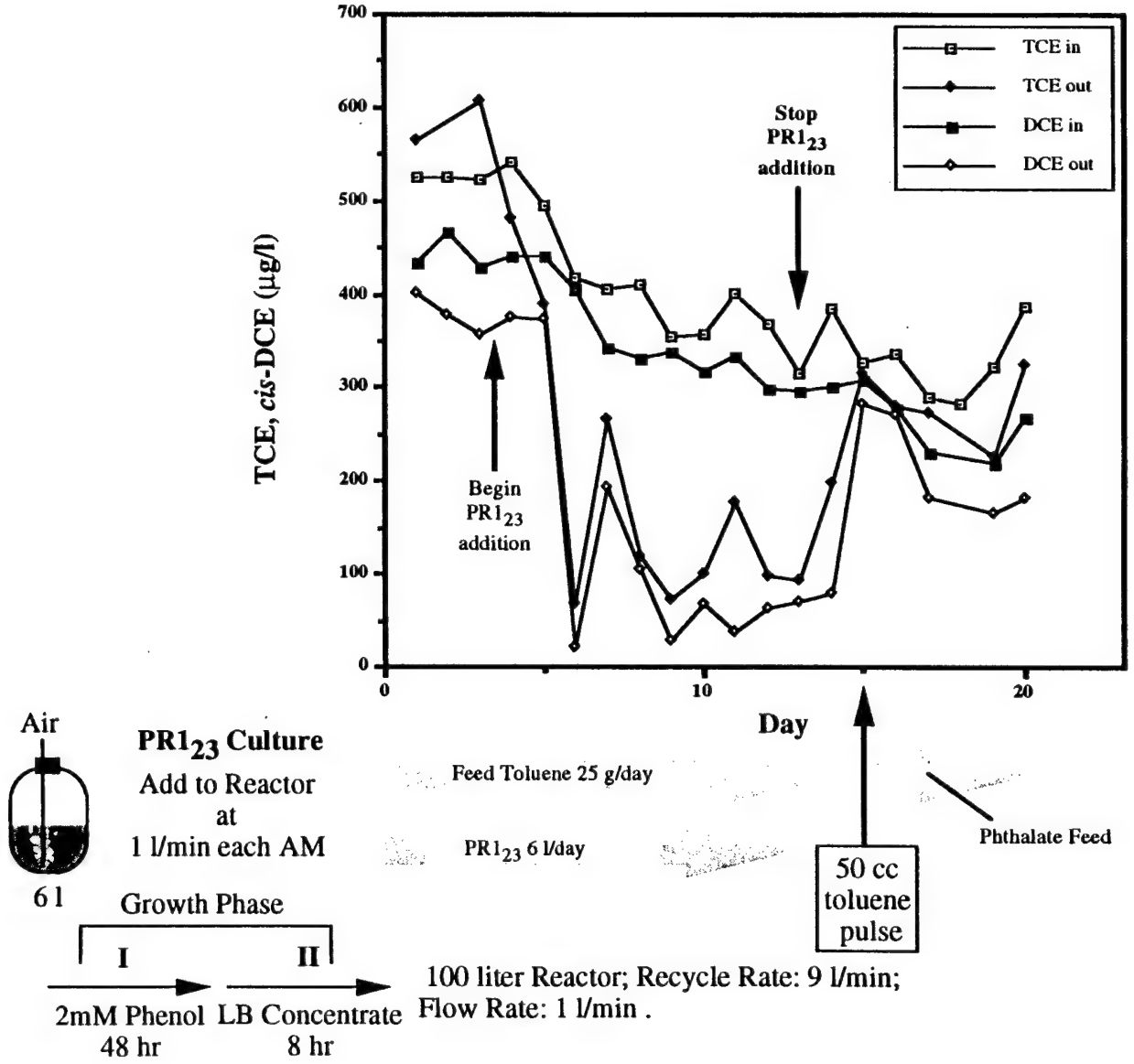


Figure 33. Summary of Cell and Carbon Additions, and Chloroethene Degradation by the Diatomaceous Earth/PR1$_{23}$ Reactor

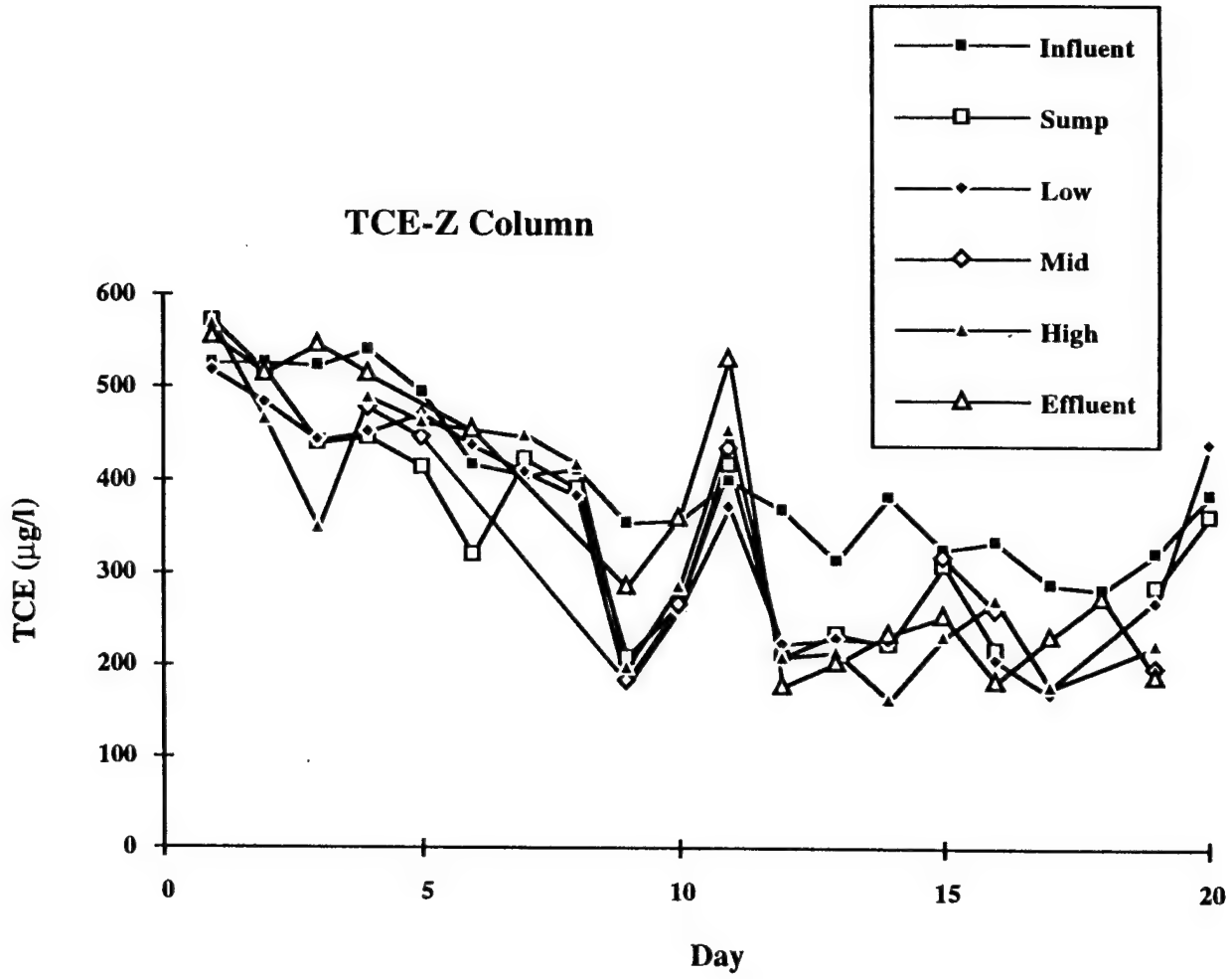


Figure 34. Aqueous-Phase TCE Degradation at All Levels in the Type-"Z" Support Column Inoculated with $PR1_{23}$

of toluene monitored in the diatomaceous earth pellet column on days 3 (before toluene addition) and 11 and 12 (when the monitored toluene concentrations were higher than usual) are given in Figure 35. The toluene spike that occurred on Day 15 due to the instant addition of 50 cc of neat toluene was not monitored because the column was drained and flushed with fresh water immediately. It is therefore impossible to determine the actual toluene concentration $PR1_{23}$ was transiently exposed to. It is well established that G4 is very sensitive to high toluene concentrations. It should also be noted that an apparent recovery of DCE degradative activity was taking place in the final three days of the experiment when phthalate was being fed (Figure 33).

The column was apparently functioning as a typical plugged-flow reactor with respect to toluene degradation. This is in direct opposition to how the chloroaliphatics were degraded in the same column at the same time (see analysis above in TCE and *cis*-DCE in/output).

(4). Microbiological monitoring of the liquid phase reactors

The TVC counts determined on LB plates and the population of toluene utilizers measured for both the M and Z columns were in excellent agreement (Figure 36). This is promising since the sole carbon source for colonization and maintenance was toluene.

(5). Location of Degradative Activity Within the Column

In an effort to determine the location of the degradative activity within the M-column pellets were taken from the Mid port, and liquid samples from the sump, effluent and groundwater feed. One sample of pellets was boiled and combined with an untreated groundwater sample. The various samples were then sealed in serum flasks with TCE @~200 μg/L, and incubated at 30°C, for 48 hours (containing 5 grams of pellets and 10 mL of sterile water or groundwater). The TCE remaining in the liquid was determined through pentane extraction (Figure 37). The only degradative activity seen was pellet-associated. These samples were taken at the mid point which is ~ 36 inches from the point of cell addition in the sump. This would further enforce the data that the degradative activity remaining in the column was due to cell

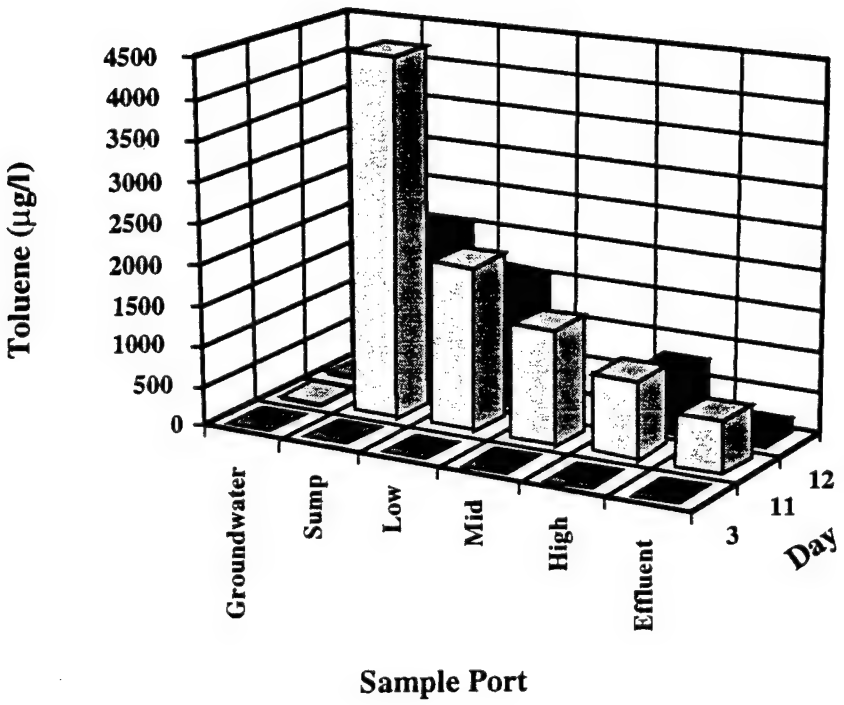


Figure 35. Toluene Degradation at All Levels in Diatomaceous Earth/PR1$_{23}$ Column

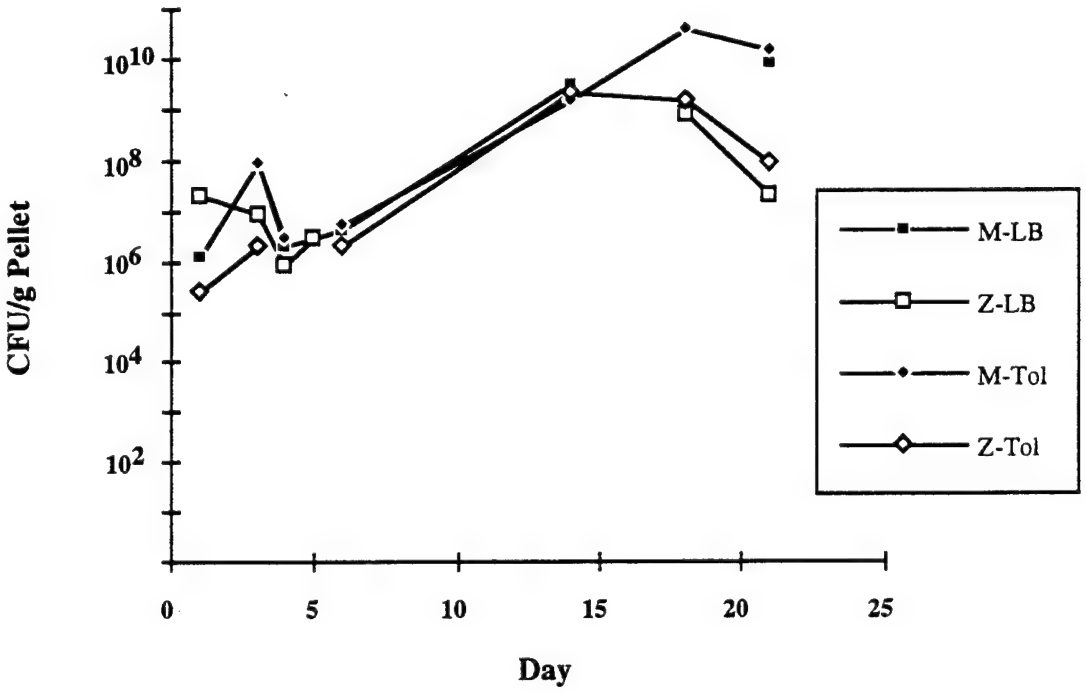


Figure 36. Microbiology of Column Packing Materials: Diatomaceous Earth (M) and Type-"Z" Pellets. Total Heterotrophs (LB), and Toluene (Tol) Utilizers

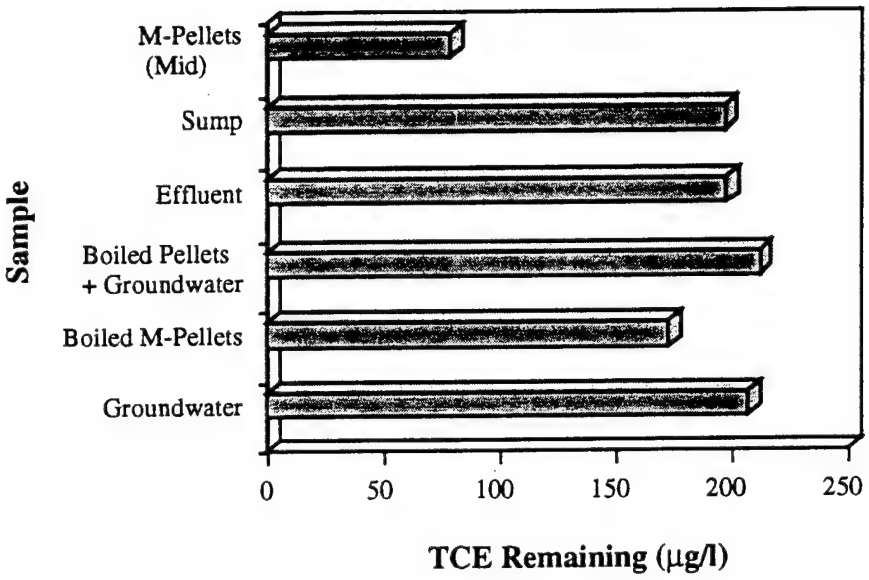


Figure 37. TCE Degradation by Individual Components of the Diatomaceous Earth/$PR1_{23}$ Reactor

association with the diatomaceous earth support material and not simply a matter of recirculating cells.

4. Tyndall Bioreactor Studies at UWF

It was decided at the conclusion of the field experimentation at Hanscom that the greatest potential existed for the utilization of the diatomaceous earth pellets under other feed conditions. Because of the continued expense of maintaining the research effort in Boston it was decided that a number of variables could now be assessed in Florida.

The goal of continued bioreactor studies at UWF were limited to efforts to increase the pellet associated biomass within the column and determine the capacity for TCE degradation without toluene or phenol. Here colonization of the diatomaceous earth reactors with $PR1_{23}$ was attempted using glucose and phthalate as carbon sources. TCE treatment was tested in the liquid phase plugged flow design used at Hanscom AFB.

(a). Colonization of the Large Bioreactors Under With Glucose And Phthalate

Colonization was followed by monitoring bacterial population densities (i.e. colony forming units [cfu]) from pellets taken from within the reactor, ground and diluted with BSM. TCE in put and output levels from the reactor, and TCE degradation by pellet samples taken from the reactor at the end of the 45 day experiment.

One of the stainless columns packed with diatomaceous earth was continuously inoculated daily with 6 liters of phthalate grown $PR1_{23}$ for 30 days. During the first four days glucose was fed to the column at the rate of 100 grams per day, and during the next six days at 200 grams per day. Phthalate was substituted for glucose as the carbon and energy source for the net thirty-five days.

(b). Microbiological Monitoring

Determinations of viable cells from pellet material were determined by grinding pellets in a mortar and pestal then and suspended this (1 gram in 1 ml) in water for serial dilutions. Populations capable of growth on LB, and

BSM containing either phenol or toluene as the sole carbon source were measured from these dilutions. Despite the daily addition of 10% of the liquid volume of the column each day as overnight $PR1_{23}$ culture, toluene and phenol degraders were usually only ~10% of the cells culturable on LB (Figure 38).

(c). TCE Degradation

The microbiological evaluations were mirrored by TCE input-output concentrations which evidenced little TCE degradative activity on days 37, 44 and 45 of operation (Figure 39). This was also demonstrated in pellet samples taken on day 45 and subjected to overnight TCE degradation assays (Figure 40).

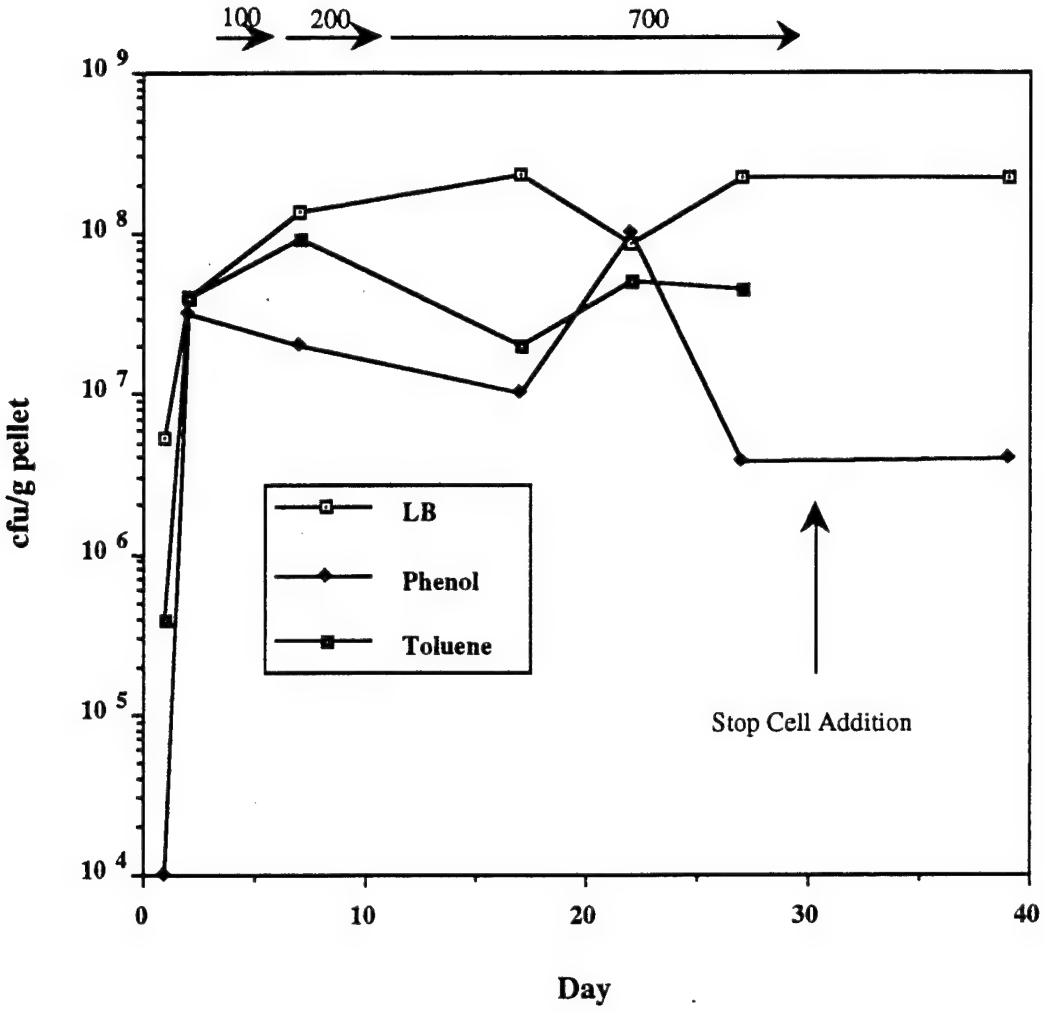


Figure 38. Microbiolgy (Total Heterotrophs (LB), Toluene and Phenol Utilizers) in the Diatomaceous Earth 100 Liter Reactor Following Glucose and Phthalate Feed

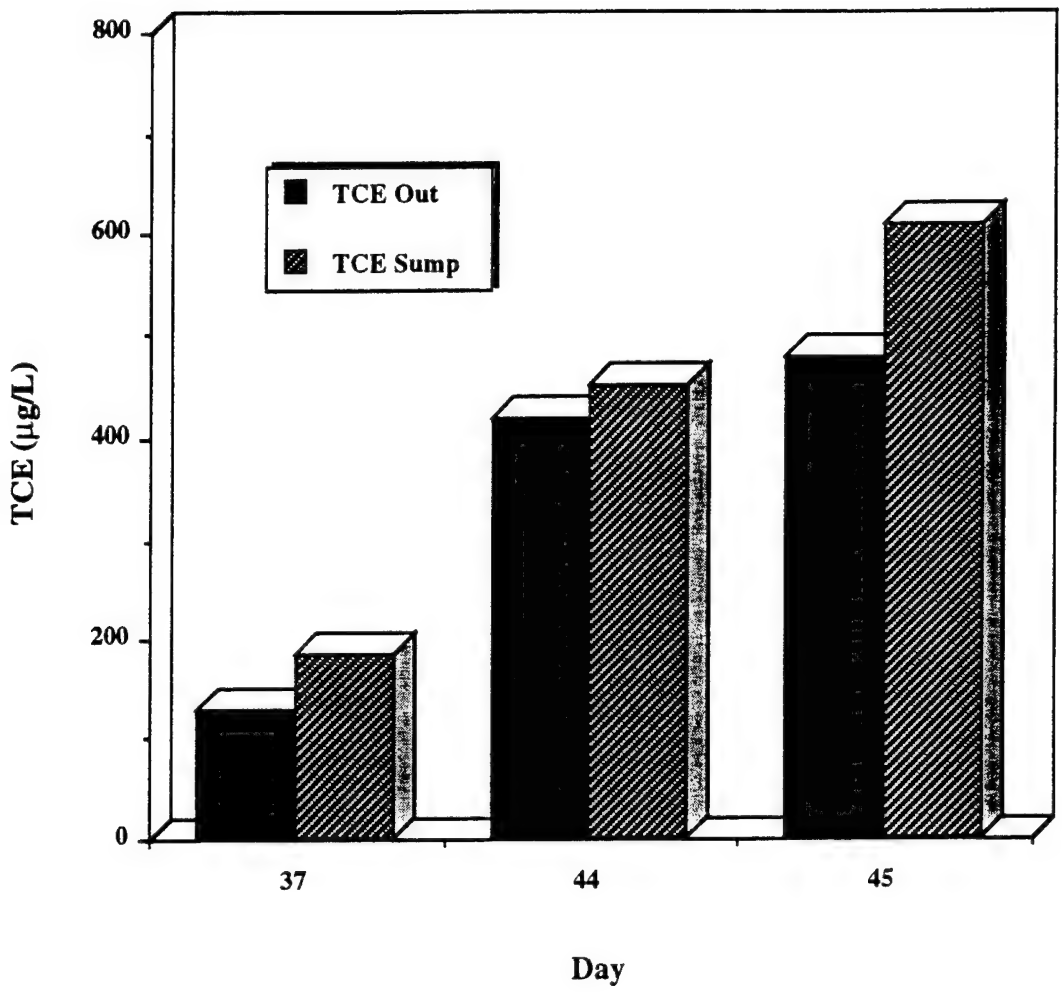


Figure 39. TCE Levels in the Input (Sump) and Output from the Glucose/Phthalate Fed $PR1_{23}$ Reactor

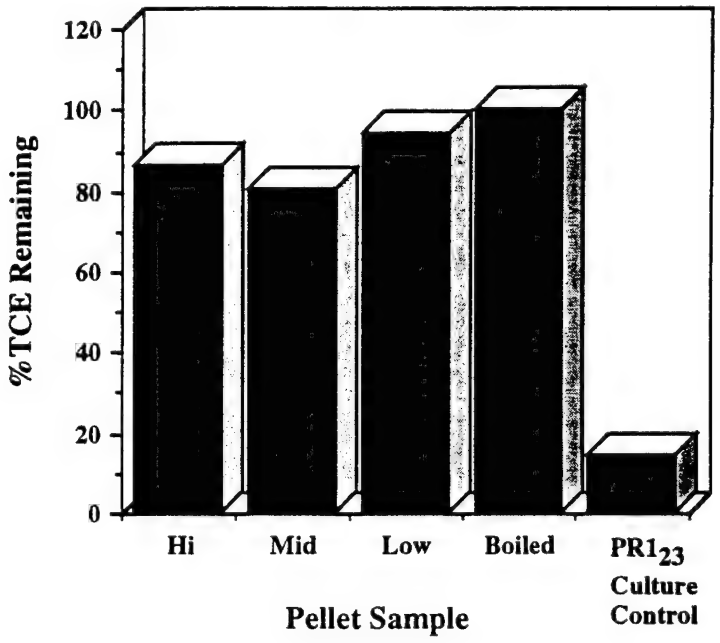


Figure 40. Overnight TCE Degradation Assay (400-500 μg/l) from the Glucose/Phthalate Reactor Components

SECTION III
NEW CONSTITUTIVE TCE-DEGRADING TRANSCONJUGANTS

A. INTRODUCTION

Following our experiences with $PR1_{23}$ in the large stainless reactors at Hanscom and again at UWF three conclusions were reached:

1. An intermediate-scale bioreactor is needed to adequately model the conditions encountered in the field with the large stainless steel reactors.
2. $PR1_{23}$ has the potential to degrade TCE under environmental conditions,
3. $PR1_{23}$ is capable of TCE metabolism under field conditions, but this strain is not sufficiently incorporated into a biofilm under the strategies employed.
4. It may be possible to take advantage of the environmentally stable phenotype of the genetic elements responsible: TOM_{23c} or TOM_{31c} if a way can be found to either:
   - a. Physically prevent the loss of $PR1_{23}$ from the reactor environment in either of two ways:
     - (1) Physical encapsulation within an inert matrix, or
     - (2) Employ a bioreactor which causes the immobilization of bacteria directly.
   - b. Transfer one of these constitutive plasmids to another bacterial strain with both the capacity to constitutively express Tom (like *B. cepacia*) as well as form an effective biofilm on the diatomaceous earth pellets under septic conditions.

For reasons of monitoring and genetic selection the remainder of the experiments were carried out with $PR1_{31}$ instead of $PR1_{23}$. This was due to information generated in our laboratory in which the genetic basis for the constitutivity in both of these strains was established. Since there is no apparent physiologically detectable difference in the two plasmids relevant to Tom expression, there are no grounds to choose one over the other on a functional basis. However, TOM_{31c} of $PR1_{31}$ harbors Tn*5*. This makes the identification of TOM_{31c} much simpler than TOM_{23c} (which does not contain Tn*5*). Transfer of TOM_{31c} to other bacterial strains can be

detected by expression of the Tn*5*-encoded kanamycin resistance or by probing for DNA sequences unique to Tn*5*.

Selection of a superior bacterial attacher was initially by means of literature review. Six candidate strains were identified as likely selectable or environmentally stable species reported to form active biofilms. These were: *Pseudomonas cepacia* 249, *Pseudomonas putida* Idaho, *Pseudomonas putida* 54 G, *Pseudomonas* sp. JS 150, *Pseudomonas putida* C600, *Pseudomonas cepacia* $PR1_{31}$.

B. METHODS AND MATERIALS

1. Organisms and Culture Conditions

$PR1_{31}$ (TOM_{31c}), 249 (TOM_{31c}) and JS150 (TOM_{31c}) were used in this phase of the study. 249 (TOM_{31c}) and JS150 (TOM_{31c}) were produced through conjugation (filter matings) of *B. cepacia* 249 (gift of Dr. Tom Lessie) and *Pseudomonas* sp. JS150 (gift of Dr. Jim Spain) respectively with $PR1_{31}$ (TOM_{31c}). Selection in both cases were for growth on BSM purified agar plates containing 2 mM phenol and kanamycin at 50 μg/mL. Even though JS150 could easily grow on phenol, JS150 (TOM_{31c}) grew much faster. 249 (TOM_{31c}) was grown on this medium at 47°C where $PR1_{31}$ (TOM_{31c}) cannot. *Pseudomonas putida* Idaho and Pseudomonas *putida* 54 G were gifts of Robert Sharp, and Pseudomonas *putida* C600 was obtained from Dr. Tamar Barkay.

2. Design Considerations For Plugged Flow Bioreactors

The addition of oxygen to the bioreactors in the laboratory represents a particular problem because of the volatility of the TCE. Since it is so volatile, any transfer of gaseous oxygen into the bioreactor will ensure a poor mass balance as the gas leaves the bioreactor. To overcome this difficulty oxygen was added to the site water by means of an oxygen saturated water stream. The principle for this is simply the counter current flow of oxygen and tap water (unsaturated) in a cylinder packed with diatomaceous earth pellets. The pellets present a large surface area and allow distribution of oxygen to very high concentrations in the water (i.e. ≥ 25 μg/L).

3. Polyurethane Entrapment of Cells.

A water containing polymer was generated from the commercial product Hypol 2000 (source??) by mixing 2.5 g of the prepolymer with 3-4 mL of an aqueous solution of cells, and allowing polymerization to occur on ice. The release of CO_2 during polymerization causes a very low density polymer to developed with a great deal of entrapped gas, water and bacterial cells.

C. RESULTS AND DISCUSSION.

1. Intermediate Scale Laboratory Bioreactor.

The use of the large stainless reactors was considered to be difficult and expensive for the testing of the research approaches listed above needs. The original 22 l glass column reactor was redesigned (Figure 41) to accommodate these diverse research needs.

The goals of the remainder of the bioreactor studies at UWF were limited to efforts to increase the biomass within the column and determine the capacity for TCE degradation without toluene or phenol. This means that in addition to colonization studies, the incorporation of immobilized or entrapped cells also had to be considered. For these reasons, the column was designed to be used as either a fluidized bed or a plugged flow reactor. It was found that the fluidized bed aspect could be accomplished through using one of the progressing cavity pumps in an enlarged recycle line. This was determined to be sufficient for a 22 L/min recycle of liquid that would perpetually maintain polyurethane (cast into 2x2 cm) cylinders in suspension over the length of the column.

The addition of oxygen to the column was a substantial problem due to the ease with which TCE is stripped by free gas within the column. Oxygen availability was largely solved through the use of a separate water oxygenation system. This consisted of an air-stripper column operated in which the aerated water leaving the column is highly oxygenated (Figure 42). Pure oxygen is introduced at the base of a column packed with the diatomaceous earth pellets. Over this column is trickled the water supply for the reactor, at a rate where 95% of the column is wetted but not saturated. The water is collected in a reservoir at the base of the column (with a continuous overflow of excess water) and pumped via a peristaltic pump to the base of the column. TCE and nutrients can be added to the line then to a static mixer before their introduction to the column. In this

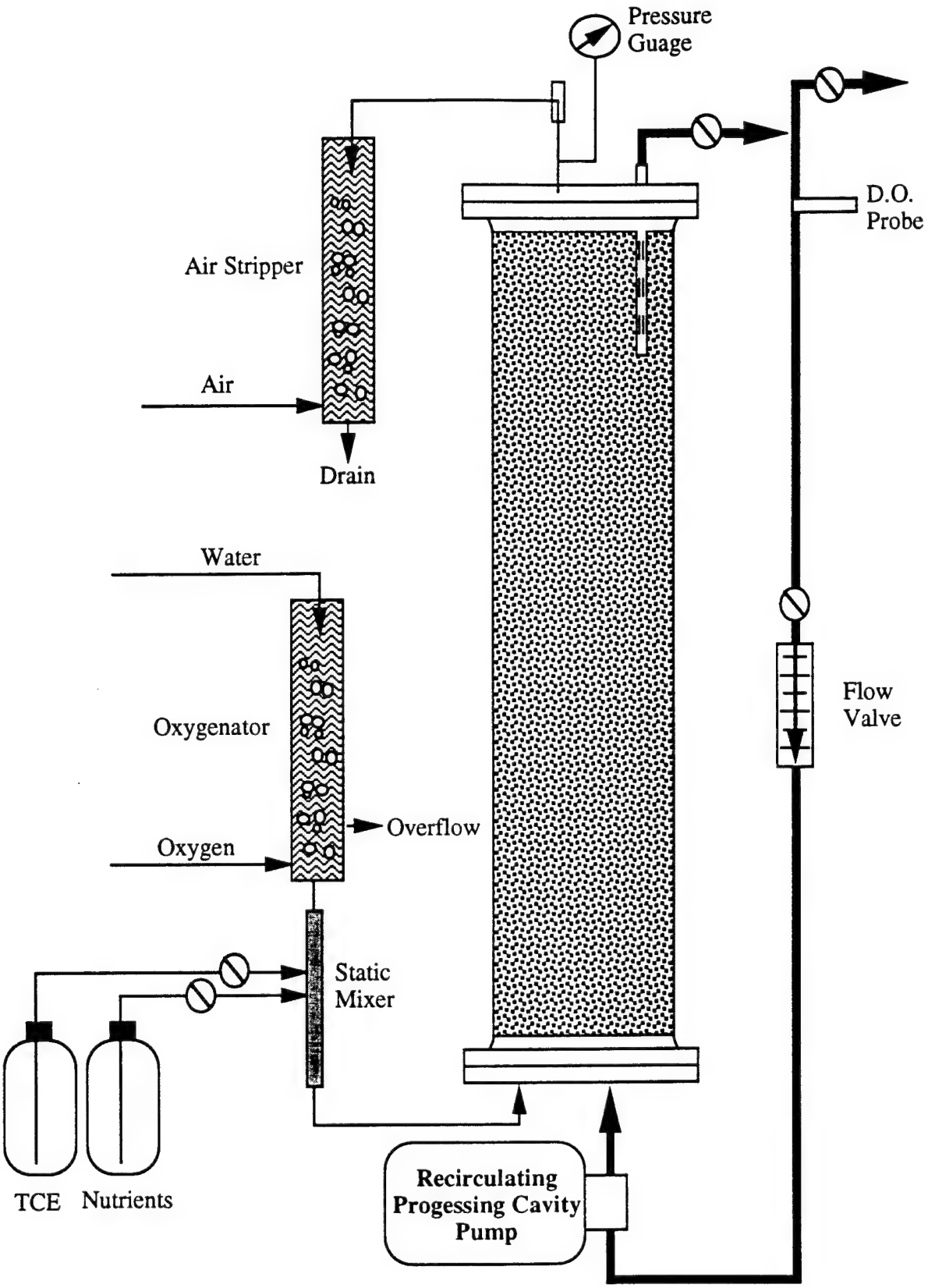


Figure 41. Diagram of 22-Liter Scale Reactor

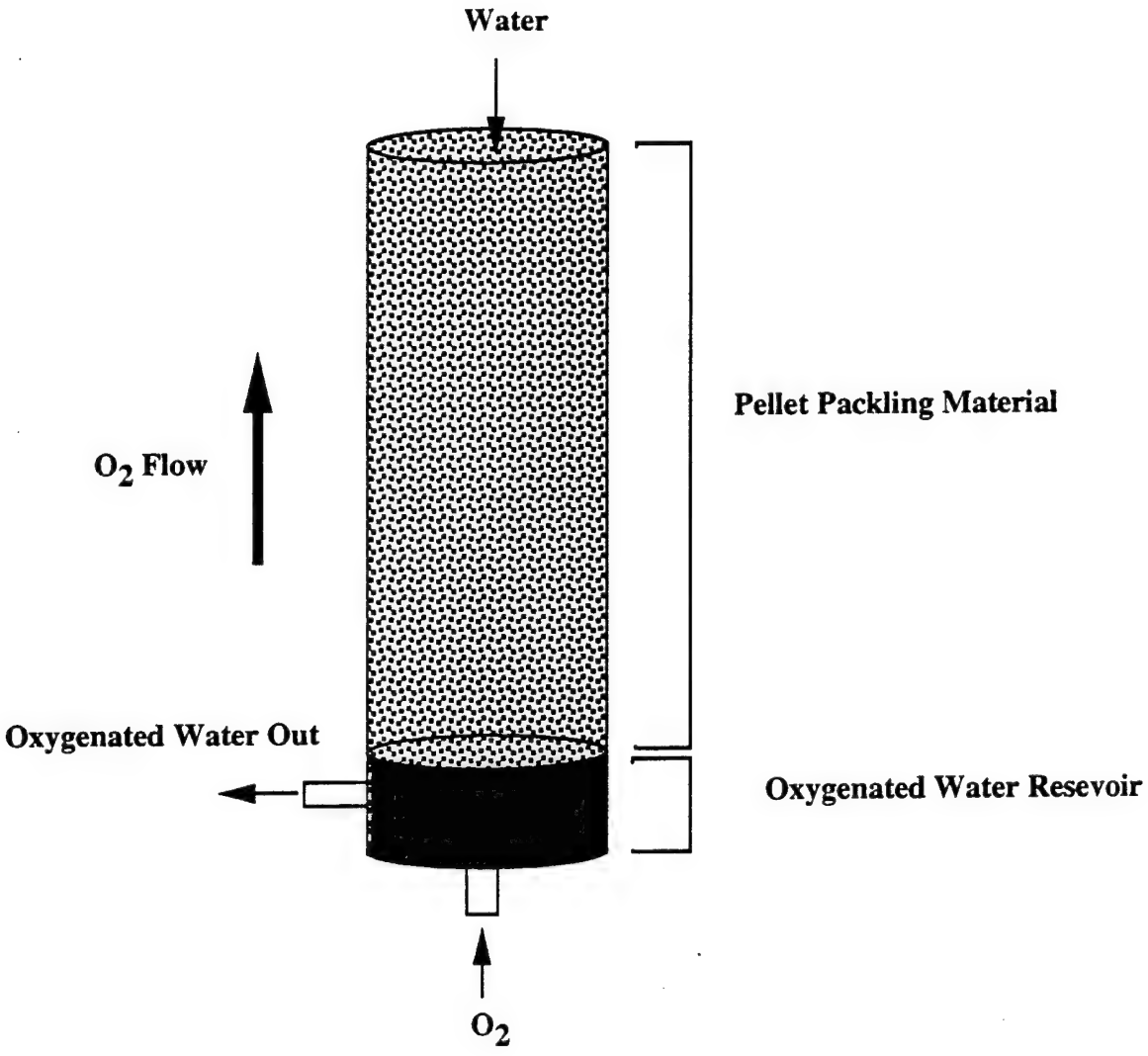


Figure 42. Diagram of Passive Oxygenation System for 22 -Liter Reactor

way, oxygen levels in excess of 25 μg/L can be attained in the influent water without stripping any of the added TCE. As long as the column is run at slight positive pressure (~5 psi) none of the gas comes out of solution in the column, thus preventing any air space for volatile partitioning. The disadvantage to this system is that site contaminated water may not be oxygenated in this manner as it will remove the volatile contaminants.

2. New Biofilm Proficient Constitutive TCE Degraders.

a. Strain Construction and Relevant Phenotypes

TOM_{31c}, bearing the essential TCE degrading enzyme (the toluene *ortho*-monooxygenase), and the genetic rearrangement causing its constitutive expression was conjugally transferred to other bacteria selected for their greater potential to establish an active and stable biofilm under attainable bioreactor conditions.

$PR1_{23}$ has been shown to colonize inert matrices under aseptic conditions. However it has failed to do so under competitive conditions. To obtain a bacterium with both the ability to attach to surfaces and to degrade TCE constitutively, we sought to introduce TOM_{31c} (a constitutive derivative isolated from the same round of Tn*5* mutagenesis in which $PR1_{23}$ was isolated. TOM_{31c}, unlike TOM_{23c}, contains an entire Tn*5*, thereby conveying kanamycin resistance to this replicon. This is a decided advantage for screening transconjugants (especially when some of the proposed recipients can also grow on toluene or phenol like JS150). Four targets were identified as desirable recipients for TOM_{31c}. They were chosen for their potentially superior performance in reactor biofilms.

P. putida Idaho *and P. putida* R54 were chosen because of their continued use in stable biofilm reactors at Montana State University (Rob Sharp, personal communication). Strain Idaho was of special interest because it can be directly selected in bioreactors through exposure to saturating concentrations of xylenes in water (Cruden et al., 1992). R54 was reported to be another successful xylene degrader, maintaining itself as a dominant member of a degradative biofilm for several months at a time.

B. cepacia 249 was reported to readily form biofilms on glass slides when fed acetate, pyruvate or succinate continuously at 1.0 mg/mL, 25°C (Murgelet al., 1991).

249 was grown in a batch reactor initially for 48 hr., then changed to 12 hour nutrient pulse per 24 hours for the next 250 hours, @ a dilution rate of 0.167/hr {32 mL/hr flow rate/ 192 mL column} during pulse) Resulted in 0.5 μg/L biomass (~60% biomass attainable in batch culture)

Pseudomonas sp. JS150 (Spain and Nishino, 1987) was selected for its capacity to utilize chlorobenzene as a sole carbon and energy source. In theory, such a selective carbon source might allow a more effective initial colonization of the bioreactor, and, possibly, an avenue to periodically remove unwanted bacteria from the biofilm and regrow the desired species.

Bacterial conjugations produced two potentially useful transconjugants: JS150 (TOM_{31c}), and 249 (TOM_{31c}). Both exhibit constitutive TCE-degradative characteristics in overnight assays , comparable to (or better than) either $PR1_{23}$ or $PR1_{31}$ (Figure 43).

b. Biofilm formation by TOM_{31c} transconjugants

To test their ability to colonize the diatomaceous earth pellets under saturated flow conditions three spinner flasks were packed with ~30% volume of pellets, and inoculated with the respective transconjugant or the $PR1_{31}$ parent. Air was continually pumped into the reactors as they would receive if run in the CSTR mode. Succinate (6 mM) was added at one of three dilution rates (based on the liquid volume [i.e. the surface of the pellets were just above the liquid]): 0, 0.55, 1.8 $hours^{-1}$ (Figure 44). The intermediate flow rate, 0.55 $hours^{-1}$) was considered to be within washout parameters as the generation time for these organisms on 6 mM succinate at room temperature was determined to be ~50 min for the fastest growing strain.

Microbiological monitoring of the pellets was done on Day 14 by plating dilutions of ground material to BSM lactate or BSM lactate kanamycin agar (Figure 45). The only strain with significantly lower kanamycin resistant counts was JS150 (TOM_{31c}). This could reflect an insignificant reading, a contaminated culture, or, more seriously, a spontaneous loss of TOM_{31c} by JS150. Only specific stability studies will answer this question. The fact that the cultures were not under the best maintenance conditions is highlighted by comparing the TFMP activities of the pellets of all three cultures on Day 8 compared to Day 14 (Figure 46).

c. Screening Primary Carbon Sources for Tom Activity of TOM_{31c} Transconjugants

249 (TOM_{31c}) and JS150 (TOM_{31c}) were evaluated for their prolonged expression of Tom specific activity (as determined through the TFMP assay) after feeding glucose, acetate or lactate as cometabolic sources of carbon and energy:

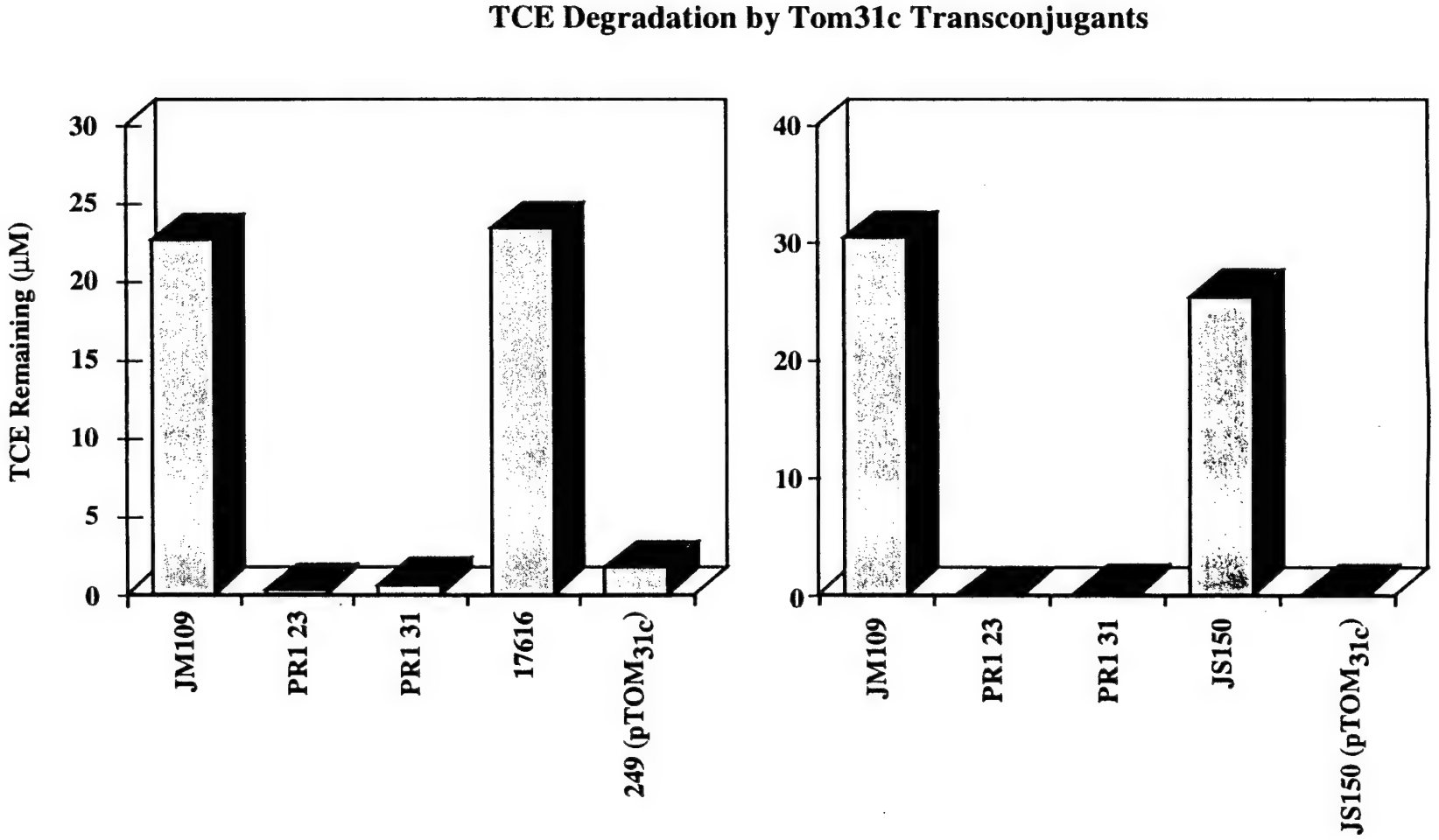


Figure 43. TCE Degradation by TOM_{31c} Transconjugants

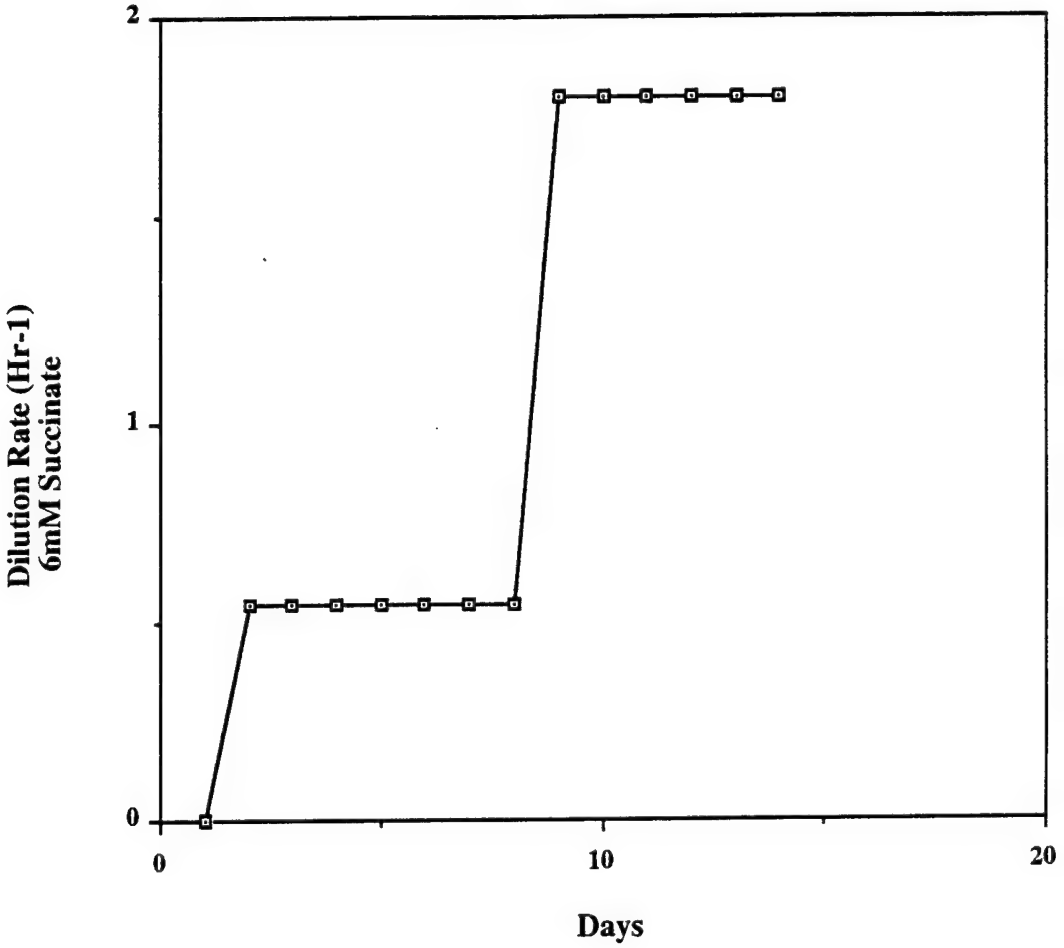


Figure 44. Rate of Phthalate Addition for Pellet Colonization

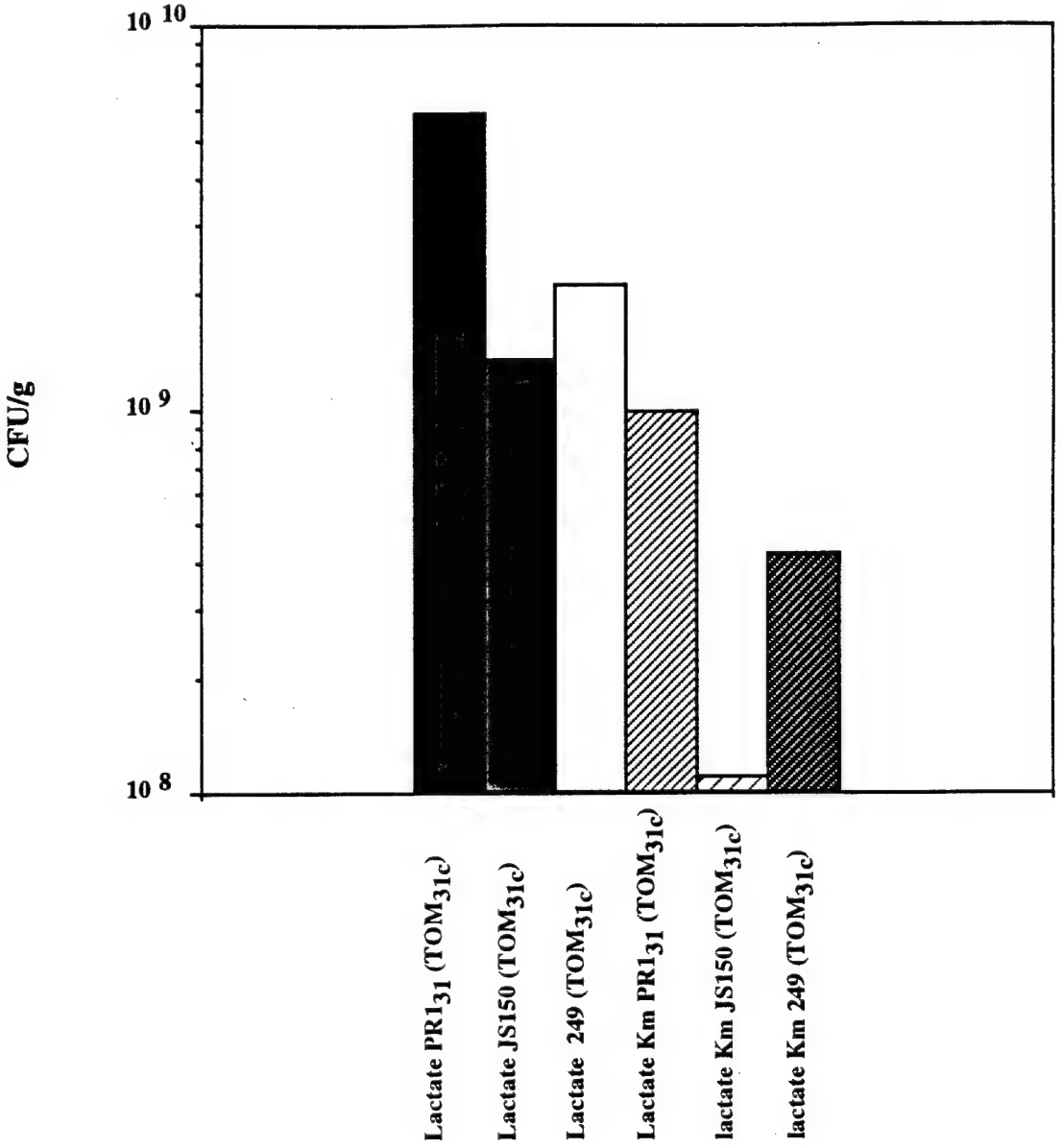


Figure 45. Microbology of Diatomaceous Earth Pellets After 14 Days Colonization

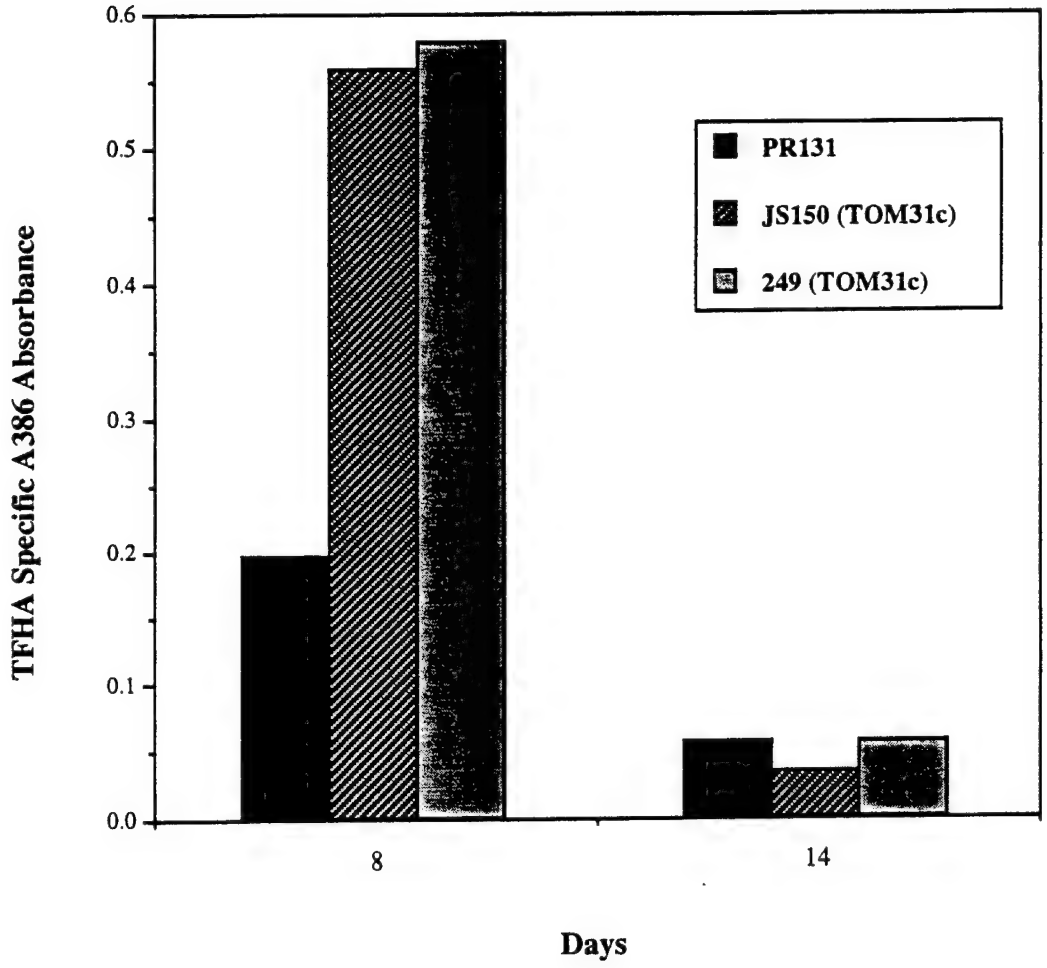


Figure 46. TFMP Oxidative Activity of the Diatomaceous Earth Pellets

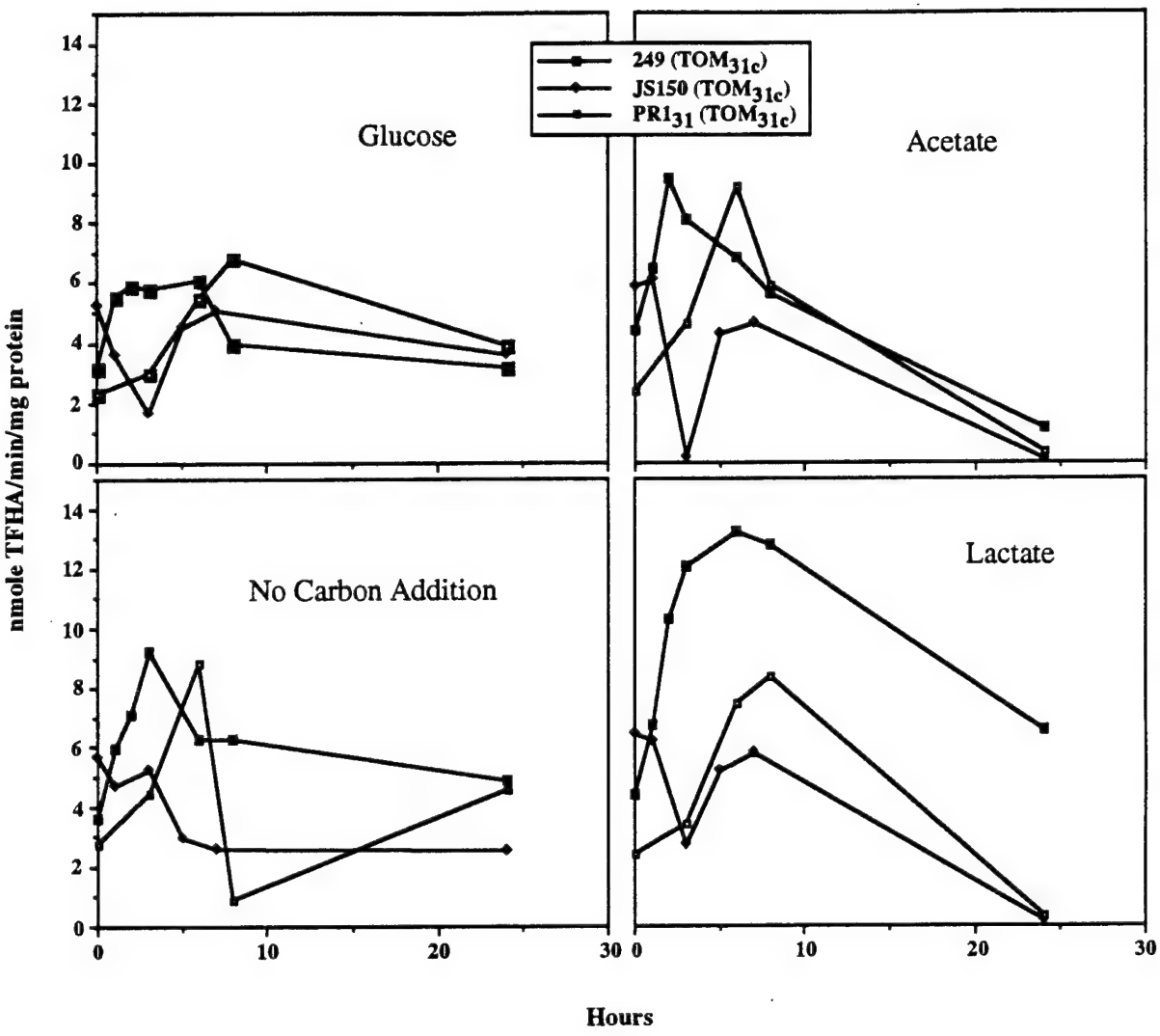


Figure 47. Carbon Sources for $PR1_{31}$ and TOM_{31c} Transconjugants and Resulting Specific Activities Towards TFMP

Figure 47 depicts the TFMP specific activity remaining to free cells of 249 (TOM_{31c}), JS150 (TOM_{31c}) and $PR1_{31}$, following feeding of glucose, acetate and lactate or nothing after growing overnight on LB medium.

JS150 (TOM_{31c}) gave a reproducible albeit unexplainable response, exhibiting a transient depression in specific activity immediately following addition of any secondary nutrient source. $PR1_{31}$ behaved as in all previous assays, not exhibiting a tremendous change in activity despite the source of energy. 249 (TOM_{31c}) however demonstrated among the highest sustained activities for all substrates tested, with a particularly good response with lactate. This is in agreement with numerous qualitative observations where 249 (TOM_{31c}) was observed to consistently have the highest and most reproducible TFMP activity.

Oxygen demands for the various carbon sources were calculated as follows:
Oxygen = 25 µg/L (O2 mw = 32 mg/mmol)
Oxygen = 25 µg/L / 32 mg/mmol = 0.78 mM in the infeed water

	mw (mg/mmol)		Molar ratios (Moles Compound: Moles O_2)
Toluene:	92	$C_7H_8 + 9O_2 = 7CO_2 + 4\ H_2O$	1/9 = 0.11
Glucose:	180	$C_6H_{12}O_6 + 6O_2 = 6CO_2 + 6H_2O$	1/6 = 0.17
Phenol:	94	$C_6H_6O + 7O_2 = 6CO_2 + 3H_2O$	1/7 = 0.14

Maximum allowable toluene concentration in reactor:

(0.11)(0.78 mM O_2) = 0.086 mmol/l (92 mg/mmol)= 7.9 µg/L

Maximum allowable Glucose concentration in reactor:

(0.17)(0.78 mM O_2) = 0.132 mmol/l (180 mg/mmol)= 23.9 µg/L

Maximum allowable Phenol concentration in reactor:

(0.14)(0.78 mM O_2) = 0.11 mmol/l (94 mg/mmol)= 10.3 µg/L

d. TCE degradation in 1 and 22 l diatomaceous earth reactors by TOM_{31c} transconjugant biofilms

249 (TOM_{31c}) and JS150 (TOM_{31c}) were tested in 1-liter plugged flow columns to evaluate the flow rates and nutrient concentrations before scaling to the larger column. 249 (TOM_{31c}) was additionally tested in the 22-liter plugged flow column.

Autoclaved diatomaceous earth pellets were colonized in stoppered 1-liter (ground glass top) graduated cylinders receiving recycled minimal salts medium to wet the surface (and changed daily). They were inoculated with an overnight 50 mL LB culture and fed toluene in the air stream passed through the column continuously. Material colonized in this fashion was then tested for its ability to degrade toluene and TCE as a plugged flow reactor receiving oxygenated water, toluene, minimal salts and TCE (Figure 48).

Results from these column studies were compared to control columns treated in the same manner except that they were neither inoculated nor precolonized with any cultured bacteria.

In general both transconjugant strains colonized the reactor diatomaceous earth pellet packing material far more successfully than $PR1_{31}$. However, colonization was done under the most aseptic conditions possible with toluene as the selective carbon source, fed in the vapor phase. While this resulted in a biofilm that gave detectable TFMP and TCE oxidation results in our standard assays, the transconjugant biofilms were apparently not sufficiently competitive with the organisms later present in the nonsterile water feed, and seemed to be soon replaced by indigenous organisms.

(1). 1 Liter reactors

The control and test 1 liter reactors were initially filled with 190 mL of 1x BSM or 150 mL of 1x BSM and 40 mL of a JS150 (TOM_{31c}) overnight LB broth culture respectively. The material was recycled at 18 mL/min with toluene added in the vapor phase at approximately 1.5 µg/L air at 0.1 l/min. This was continued for twelve Days with daily draining and replacement of the column with 0.5x BSM.

The column was converted to a plugged flow liquid design by pumping oxygen-saturated water (~25-30 µg/L) into the column in place of the air at 15 mL/min. Joining the input water line was a 40 µg/L stock of toluene in 1x BSM introduced at 1.5 mL/min. This provided a toluene input concentration c.a. 2400 µg/l. On Day 27 TCE addition was initiated through the same line at 1.5 mL/min from a water stock at 2400 µg/L.

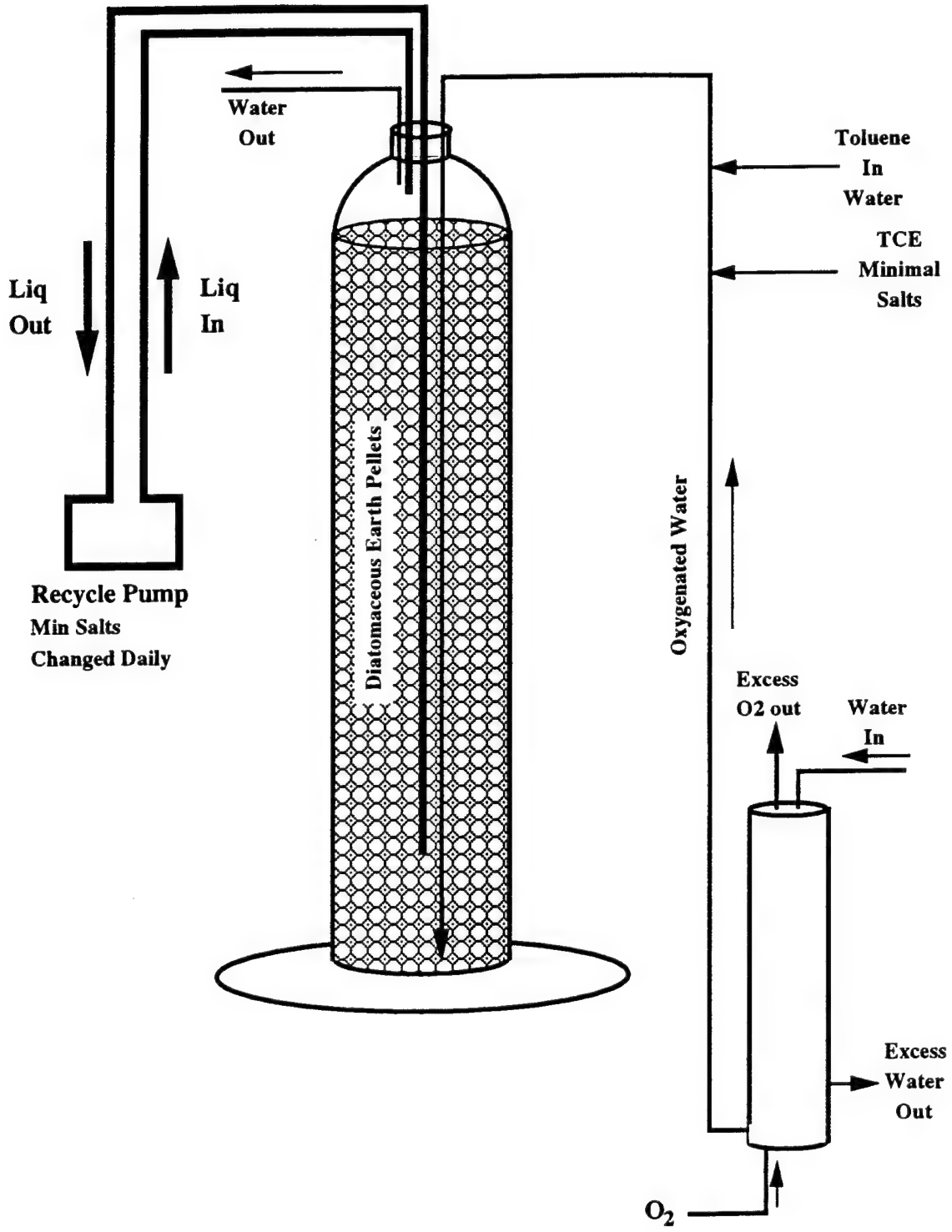


Figure 48. Diagram of 1 -Liter Graduated Cylinder Reactors

JS150 (TOM_{31c}): Aqueous samples taken from the influent and effluent ports were analyzed for both toluene and TCE via Purge-and-Trap GC (PID detector). The Reactor was run under the conditions described above for 62 Days with nutrient sources altered as follows:

The sole carbon and energy source for the reactor was 2.4 μg/L toluene for Days 1-35. On Day 36 this was changed to 12.5 μg/L glucose,and this was increased to 16.7 μg/L on Day 40. Phenol was substituted on Day 45 at 12.5 μg/L, and raised to 16.7 μg/L on Day 49. The column was returned to toluene feed at 12.5 μg/L on Day 50. On Day 63 the experiment was terminated and dilution plates were made from crushed pellet material. Representative colonies were pulled to nitrocellulose filters for probing with *tom* specific ssDNA sequences.

The relative efficiencies of these columns for the removal of toluene and TCE were determined and are shown in Figure 49 and Figure 50, respectively. Clearly a toluene utilizing biofilm community was established. However, as indicated by the *tom* probe hybridization results (see below), there were no detectable TOM_{31c} bearing colonies detected. This indicated that another toluene-degrading community other than the inoculated JS150 (TOM_{31c}) rose to dominance over the course of this experiment.

249 (TOM_{31c}): 249 (TOM_{31c}) was run in a manner similar to JS150 (TOM_{31c}) in another 1 liter column, colonized with toluene vapor as the sole carbon source. The 249 (TOM_{31c}) column was allowed to continue in this colonization phase for 43 Days, with a daily drain and refill of 0.5X BSM, while data were being collected on the JS150 (TOM_{31c}) culture.

On Day 43 this and an uninoculated column were started as plugged liquid flow reactors as described above. TCE was introduced on the third Day of liquid flow. The column was run for a total of 66 Days, during which time the nutrient source was changed twice. On Day 66, the experiment was terminated and dilution plates were made from ground diatomaceous earth pellet material. Colonies were pulled from the plates to nitrocellulose filters for gene probing.

The results of this experiment are given in Figure 51. TCE degradation began with a significant removal, but this was lost soon after toluene was replaced with phthalate as the carbon feed source on Day 11. TCE degradation was partially restored on Day 21 with the reintroduction of toluene.

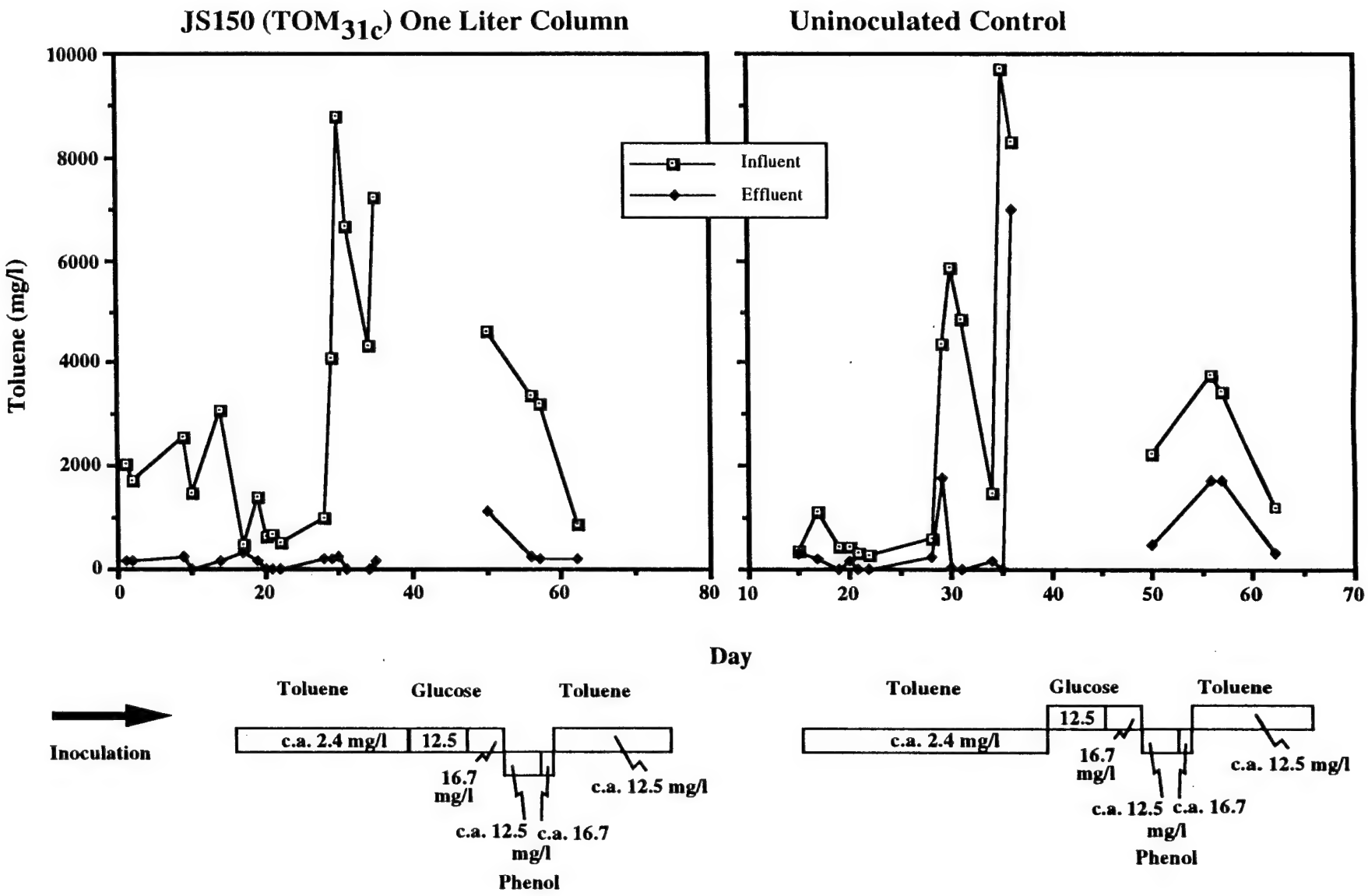


Figure 49. JS150 (TOM_{31c}) and Uninoculated Contol 1 -Liter Columns: Toluene Degradation and Application Schedule

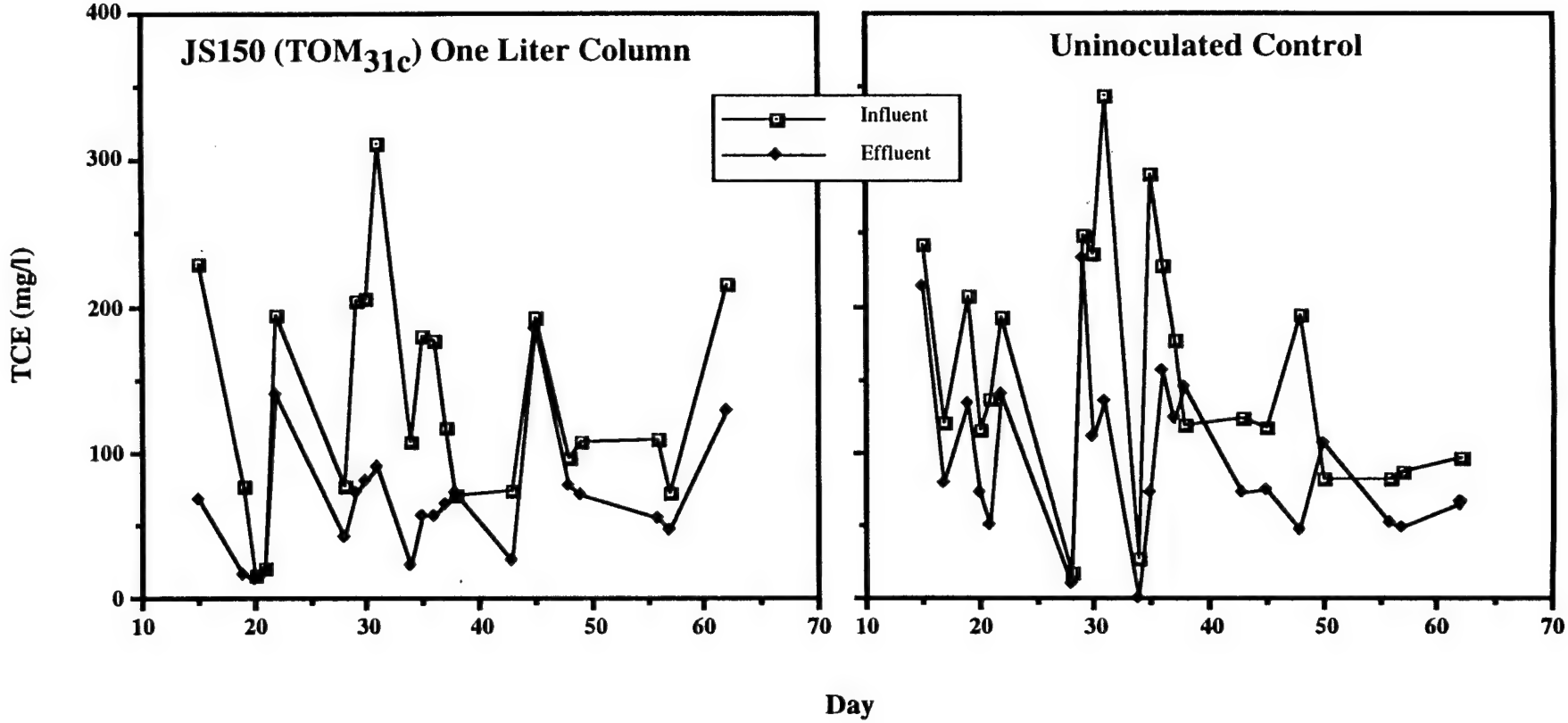


Figure 50. TCE Degradation by JS150 (TOM31c) and an Uninoculated Contol 1 -Liter Columns

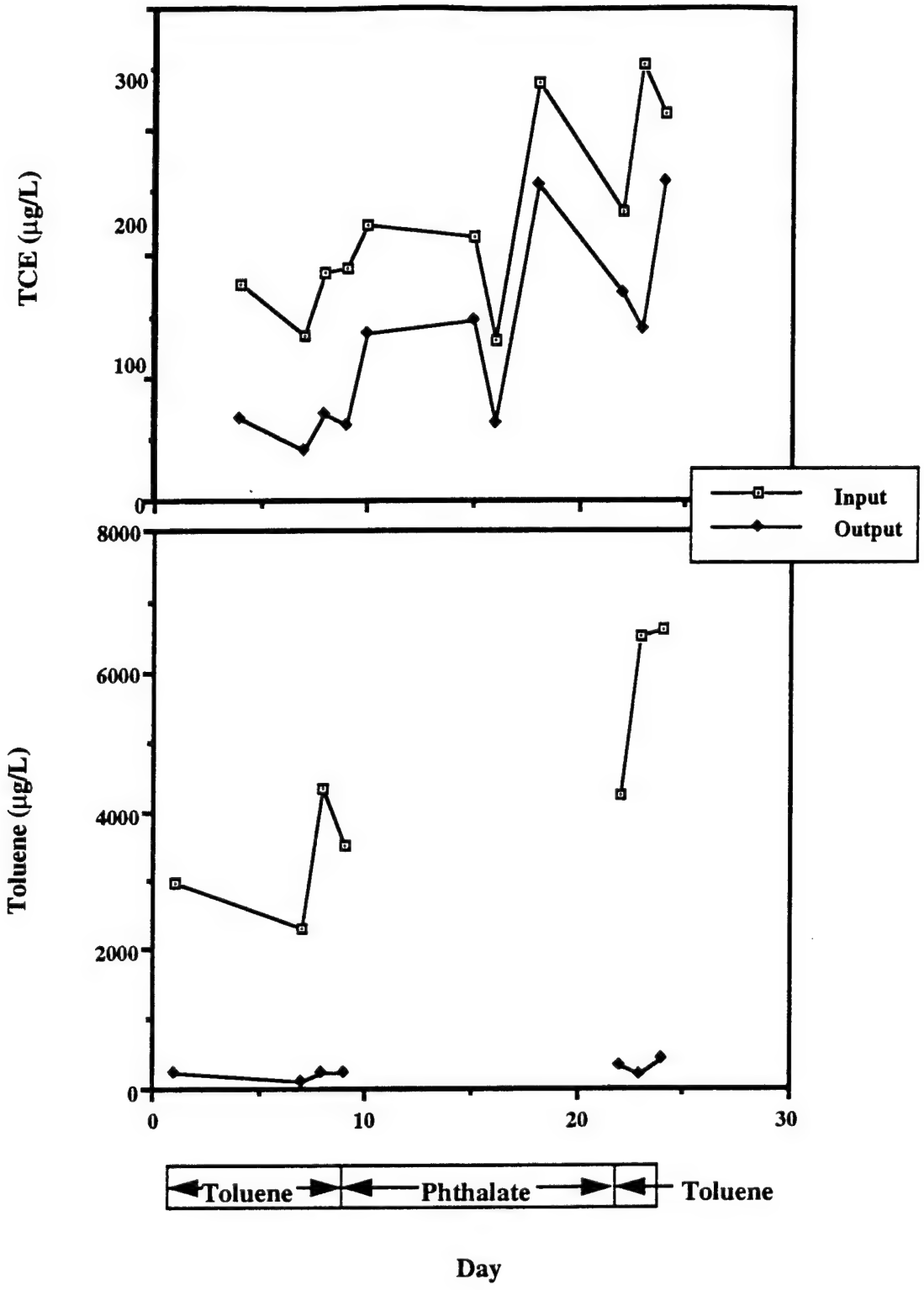


Figure 51. 249 (TOM31c) and Uninoculated Contol 1 -Liter Columns: Toluene and TCE Degradation and Carbon Source Application Schedule

These results are very similar to those seen for JS150 (TOM_{31c}). Our interpretation of the data is that despite an early presence and activity of introduced cells in these bioreactors (apparently as a biofilm) they fail to maintain themselves as the predominant species. Even though the two new transconjugant strains: 249 (TOM_{31c}), and JS150 (TOM_{31c}), were chosen for their capacity to form a biofilm, they have only been studied in this capacity as relatively pure cultures. JS150, even though originally used by Spain an co-workers for the catabolism of chlorobenzene also found that under field contamination conditions, the JS150 culture was rapidly replaced with more aggressive colonizers and degraders.

It appears that we have now done this for three species with three different carbon sources. The establishment of a co-metabolic biofilm community may not be possible working from the direction we have taken thus far: that is construction of a superior TCE degrader first, and then trying to develop a bioreactor system that will encourage its dominance.

(2). 22 l Reactor

JS150 (TOM_{31c}): At the same time preliminary data was being collected with the smaller reactor (mid-February) the 22 l column, packed with diatomaceous earth pellets was inoculated with 1 liter of an overnight culture of JS150 (TOM_{31c}) and 6 liter fresh LB glucose. Toluene was introduced in the air stream the next Day. Three liters of the 0.5 X BSM recirculating buffer was replaced daily. The carbon source was switched to chloro-benzene vapor after 6 Days. First Day chlorobenzene concentrations were measured at 66.8 µg/L air. These were reduced to 5.5 µg/L air and maintained at this level for 4 Days. No evidence of JS150 (TOM_{31c}) growth was detected in the column during this time period. No biofilm was detected during the first attempt at colonization through chlorobenzene feed as determined through direct observation,and our failure to detect oxygen uptake in the 22 liter reactor when sealed.

Because concurrent experiments indicated that toluene was serving as a good carbon source for the JS150 (TOM_{31c}) inoculated 1 liter reactor, the 22 liter reactor was changed to toluene vapor as the input carbon source (1.38 mg toluene/l air) as well, and reinoculated with 1 liter of an overnight JS150 (TOM_{31c}). These conditions were maintained for 22 Days, with 2 liters 0.5X BSM liquid volume replacement per Day. On the 23rd Day the column was changed to a liquid plugged-flow conditions. Water was

passed through the column at 75 mL/min (with a 9 L/min recycle) containing toluene and O_2 at 0.54 and ~25 µg/L and BSM to 0.1X. After 6 Days TCE was added to the water stream c.a. 500 ppb.

Pellets were taken on March 7, 1994 (immediately before changing to toluene feed) and March 13 (6 Days after reinoculation and the reinstitution of toluene feed), ground and diluted in sterile buffer for the microbiological determinations given in Table 4.

Table 4. Microbiology of JS150 (TOM_{31c}) column test.

Date	Event	Total Viable Count		
		Phenol	LB kanamycin	LB Glucose
3/7/94		3.09×10^7	1.75×10^8	2.25×10^8
	% TFMP Positive Colonies	99%	93%	80%
3/8/94	Reinoculate 1 L fresh JS150(TOM_{31c})			
3/13/94		$<1 \times 10^6$	1.12×10^7	1.43×10^7
	% TFMP Positive Colonies	$<1 \times 10^6$	27%	4%

JS150 (TOM_{31c}) was apparently maintained within the pellets from February 17 - March 7. During this time it appeared that JS150 (TOM_{31c}) was establishing itself with chlorobenzene fed in the vapor phase as indicated by the close agreement between total heterotrophic counts (2×10^8) and the more selective LB kanamycin counts (1.75×10^8). This is further supported by the phenol-degrader count: 3.09×10^7. This is especially promising since there was no selection for phenol utilization by the bacteria in this column, whereas JS150 (TOM_{31c}) is capable of doing so. The colonies that grew on phenol did so at a rate typical for JS150 (TOM_{31c}) (which is noticeably faster and larger colonies than JS150 which also supports the conclusion that TOM_{31c} was maintained through this period).

These data, however, contradict to our daily attempts to measure oxygen uptake in the sealed column receiving chlorobenzene. Apparently, the bacterial numbers and resultant total activity were insubstantial in terms of the oxygen uptake. The chlorobenzene did seem to be effective in maintaining JS150 (TOM_{31c}) at a low cell density without apparent invasion by other bacterial colonizers during this period.

Following a reinoculation with JS150 (TOM_{31c}) on 3/8/94 and changing to toluene feed, the total viable count dropped to 1.43×10^7 in 13 Days (an overall 94% reduction in bacterial biomass culturable on LB glucose). This was mirrored by the reduction in kanamycin resistant cells (and was also seen in the phenol plates which failed to give countable numbers of colonies i.e., only 2 colonies at the 10^{-5} dilution [the lowest dilution plated]).

The behavior was what one would expect from a toxic input to the column. Despite relatively high pH problems with the column in January, none were detected during this test period despite continual monitoring. Also oxygen could not have been limiting as the column was vapor fed toluene in an air stream during this time.

The column was changed to total liquid throughput on March 31. Toluene stock solution at 12,500 µg/L in 2.5 X BSM (@3mL/min)(Days 1- 17, 6,000 µg/L Days 18-20, and 2,000 µg/L on Days 21-34) was introduced, along with TCE as a stock at 12,500 µg/L in water(@3mL/min); and oxygenated water (with pure O_2 to ~ 25 mg O_2/l) (@69mL/min) giving a total liquid flow rate of 75 mL/min, and a liquid detention time in the column of 80 min without recycle. Recycle was maintained at 9 l/min. The toluene and TCE input and output levels from this column were monitored for the next month (Figure 52).

TCE measurements in the output water were extremely variable. This is in marked contrast to the stability of the input measurements. At present, we are at a loss to explain these apparent fluctuations. Whatever the source, the effect seemed to moderate after Day 20. Relatively little TCE was removed during this period, while toluene degradation was very good as the bacteria adapted to toluene as the carbon source.

While TCE degradation did occur in the column during this time, it was concluded (partly on the basis of the microbiological testing and partly due to the experiments with the 1 liter column where toluene was removed as the input carbon source) that such degradation was not due to the constitutive Tom expressing JS150 (TOM_{31c}) originally added to the column.

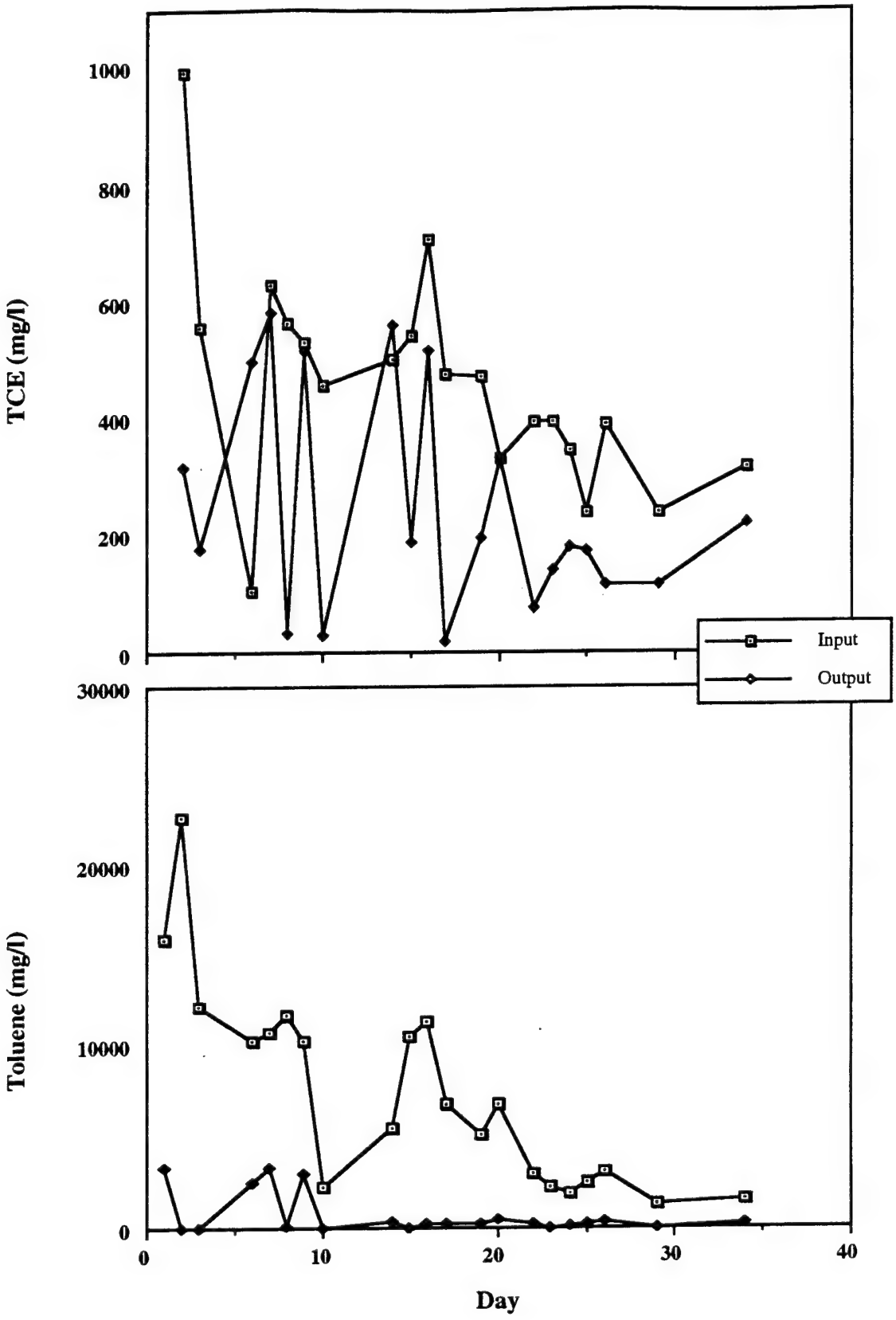


Figure 52. Toluene and TCE Degradation by JS150 (TOM31c) in the 22-Liter Reactor Packed with Diatomaceous Earth Pellets

249 (TOM_{31c}): At the end of May, 1994 the 22 liter column was cleaned and inoculated with 249 (TOM_{31c}) using 12.5 mM phthalate as a carbon source. This was done because of the immediate preceding failure of JS150 (TOM_{31c}) to be selectively maintained with toluene as the carbon source.

Initial results indicated a loss of 39-47% in output concentrations of TCE relative to input. Oxygen demand in this column was measured at 3.6 mg O_2/l/hour.

TCE degradation relative to the input water,, over the following week, however, fell to an average of only 19%. This was not considered significant and this reactor experiment was terminated.

(3). Radioactive DNA probes for biofilms of 249 (TOM_{31c}) or JS150 (TOM_{31c})

To determine if the organisms modified with TOM_{31c} (249 (TOM_{31c}) or JS150 (TOM_{31c})) were capable of stable colonization of the diatomaceous earth pellets with toluene as the sole carbon source, colonies obtained from BSM toluene plates were screened by colony hybridization techniques with a ^{32}P labeled 58 kb fragment of TOM_{31c} known to encode the toluene degradative pathway. Colonies grown on these toluene selective plates should represent the actual toluene degrading biofilm present in the columns. Detectable hybridization among these colonies to the 58 kb TOM_{31c} fragment would indicate the proportion of the biofilm that is either the cells we added (i.e. 249 (TOM_{31c}) or JS150 (TOM_{31c}) or at the very least another bacterial strain that contained a plasmid similar or identical to TOM_{31c}.

Positive and negative control colonies ($PR1_{23}$ and *E. coli* respectively) yielded the predicted hybridization patterns. Colony counts indicated a concentration of between 1 and 5 x 10^7 toluene utilizers per gram of diatomaceous earth substrate material. Approximately 100-200 colonies from each column grown on BSM toluene were pulled and probed.

None of these bacterial colonies (grown from the 249 (TOM_{31c}) or JS150 (TOM_{31c}) inoculated columns) hybridized to our TOM specific probe. These results indicate that despite selective carbon source utilization (toluene) neither of the constructed strains were competitive with indigenous bacteria also capable of growth with toluene.

It is of interest to note that these toluene induced bacteria were fully capable of TCE degradation when toluene was present. Obviously the native toluene degradative

capabilities we inadvertently selected for are not those encoded by the TOL/XYL encoded pathways since toluene induced TCE degradation.

3. Physical Encapsulation of $PR1_{31}$ in Inert Matrices.

Polyurethane and calcium alginate were tested as immobilization matrices for $PR1_{31}$ for use in a fluidized bed bioreactor.

a. Polyurethane Encapsulation

The first encapsulation matrix tested was polyurethane. Polyurethane "entrapment" of bacteria, while fast and reasonably effective in the fluidized bed system proved to be less than efficient in actually preventing the loss of $PR1_{31}$ by dilution.

Unlike the calcium alginate polymer, the PU polymer is not actually an "encapsulation" technology. It is meant more to be an "entrapment matrix" wherein the bacteria should be able to regenerate their dilution losses in a bioreactor. This was attempted with $PR1_{31}$ so "entrapped" for 300 hrs in a fluidized bed mode of operation. The pH, dissolved oxygen (DO) and temperature are given in Figure 53. The DO in relation to phthalate feed concentration (c.a. levels entering column in the water infeed) and column flow rate (throughput) is given in Figure 54. Apparently the column was operating above washout conditions at the higher flow rate. This was confirmed by lowering the flow rate 90%. This resulted in turbid growth in the reactor within 125 hrs of operation. This was also concomitant with the highest oxygen demand. The turbidity was lost upon doubling this flow rate to 50 mLs/min, but the oxygen demand remained high for the next 100 hrs nonetheless. This might be interpreted to mean that residual phthalate degrading bacteria within the PU matrix resisted washout. Oxygen demand at 310 hours (determined by sealing the reactor for one hour and monitoring oxygen levels in the recycle line) was not detected. Microbiological monitoring of this column was by plating to BSM-phthalate plates, and subsequent determination of the proportion of the resulting colonies that constitutively converted TFMP to TFHA (Figure 55). The only significant reduction in TFMP positive colonies was seen from dilutions made at the last data point, where they appear to have become suddenly and completely eliminated. While there is no particular reason to doubt this particular datum it is surprising in its suddenness. it. It is possible that the transient extremely low oxygen concentration selectively eliminated $PR1_{31}$ from the matrix, allowing the smaller fraction of TFMP negative cells to proliferate. If this is the case, it would be prudent to examine the more "encapsulatory" matrices from not only the standpoint of prevention of $PR1_{31}$ washout, but for exclusion of competitors as well.

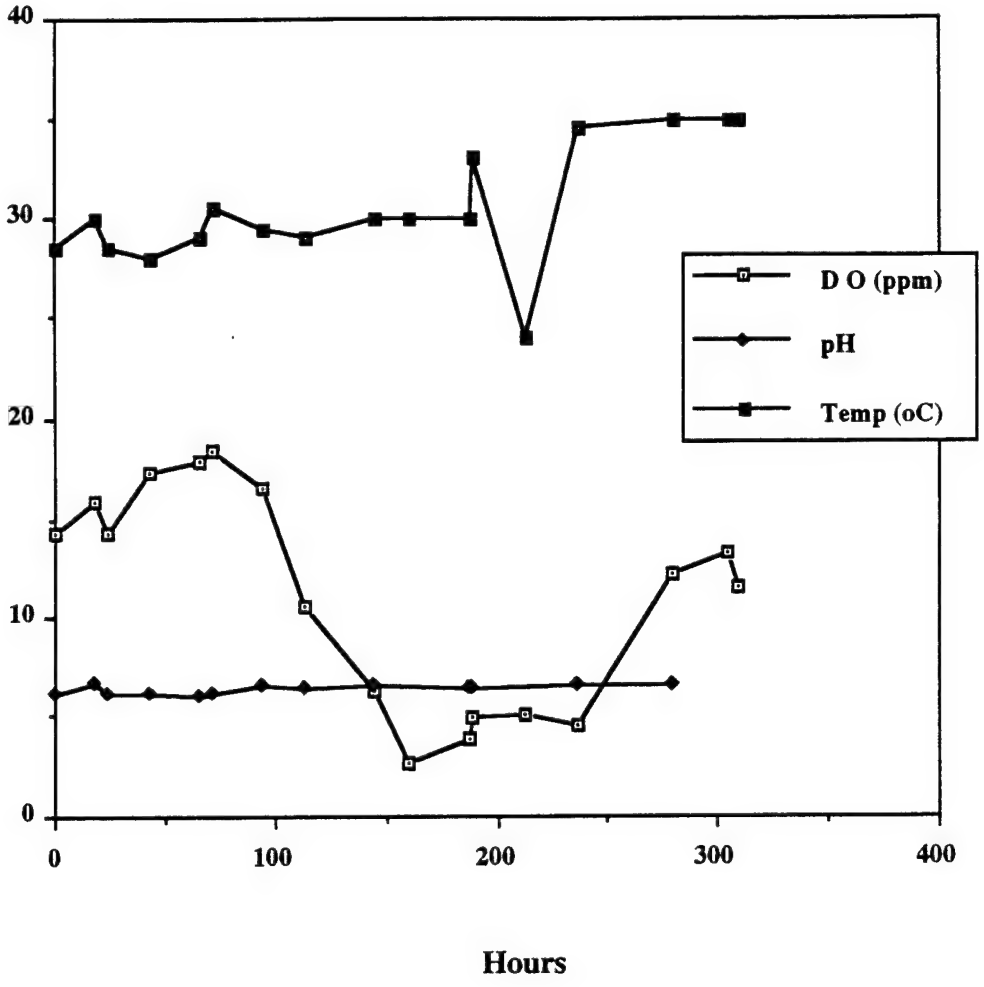


Figure 53. Dissolved Oxygen, pH, and Temperature of the Fluidized Bed Reactor Containing $PR1_{31}$ (TOM_{31c}) Cells Entrapped in Polyurethane.

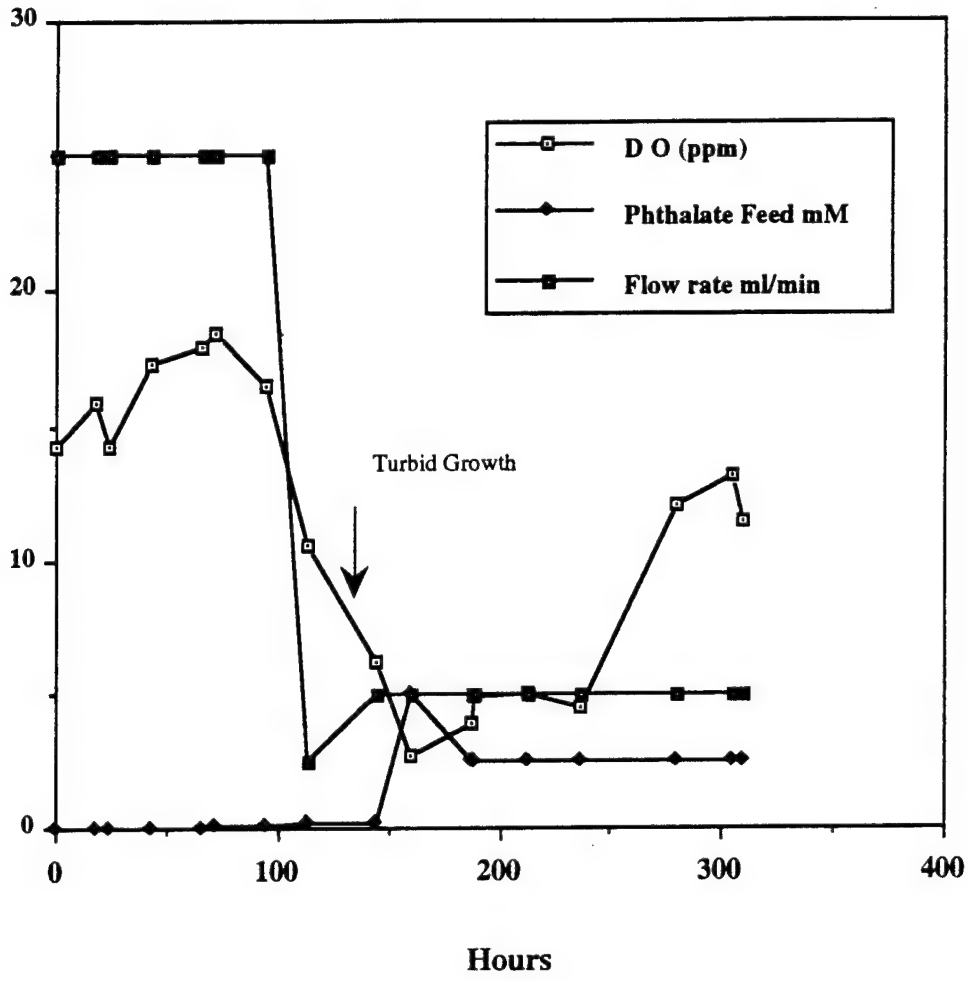


Figure 54. Dissolved Oxygen as a Function of Phthalate Feed Concentration and Flow Rate in the Fluidized Bed Reactor Containing $PR1_{31}$ (TOM_{31c}) Cells Entraped in Polyurethane

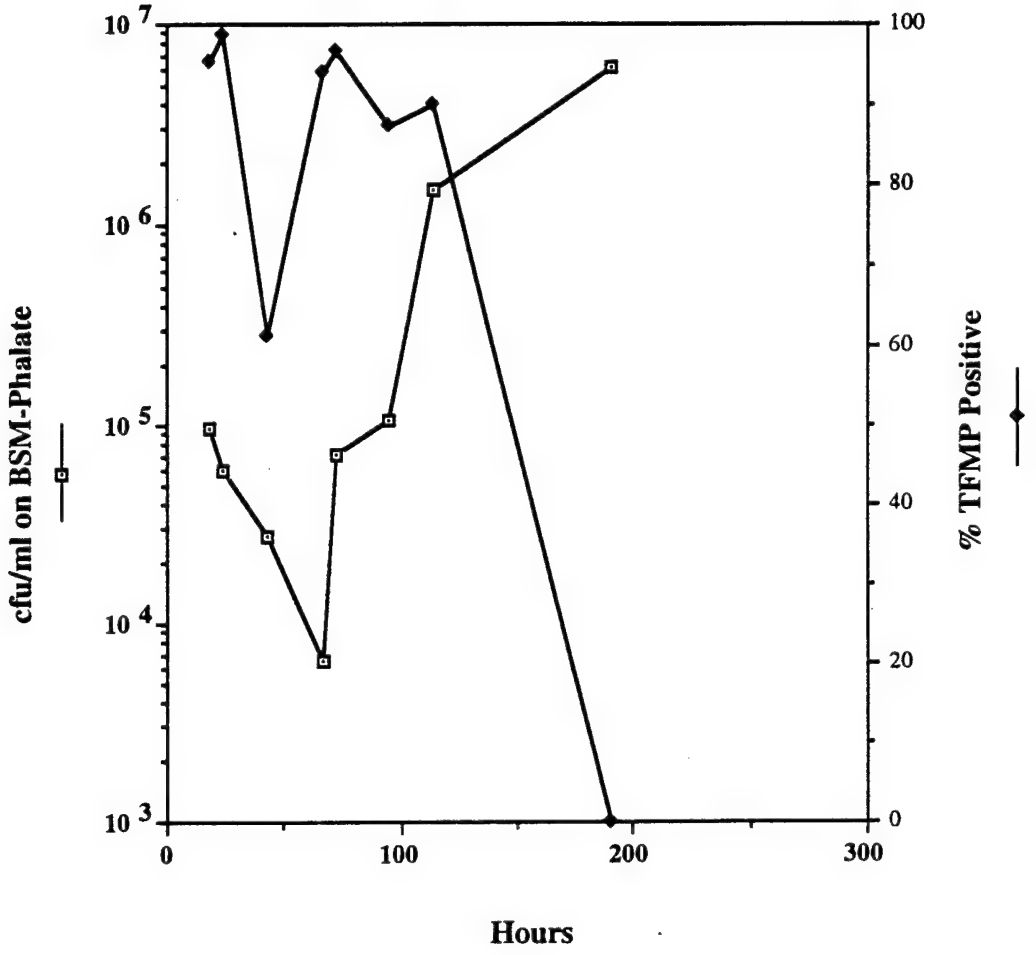


Figure 55. Microbiology of Effluent Water from the Polyurethane -Entrapped $PR1_{31}$ (TOM_{31c}) 22 -Liter Fluidized -Bed Reactor.

b. Alginate Encapsulation

The ability of 249 (TOM_{31c}) to effectively metabolize TFMP under a variety of exposure conditions: including TCE exposure, minimal C:N:P ratios and limited reducing source is shown in Figure 56. 500 ppb TCE does not apparently impair Tom activity towards TFMP where as 3 mM peroxide and reduction of the concentration of the lactate carbon source by 97.5% clearly do.

Shake flask experiments with alginate entrapped 249 (TOM_{31c}) were performed to determine the carbon, nitrogen and phosphorus concentrations optimal for Tom activity. In the first study seven different carbon concentrations with varying amounts of nitrogen and phosphorus (yielding C:N:P ratios of: 10:1:5; 10:1.25:1.05; 100:6.3:5.2; 100:4.1:3.5; and 100:2.5:2.1) were tested for their effects on Tom activity as measured by TFMP oxidation rates following 24 hr in these respective media. It was observed in previous trials that any phosphorus concentration above 7.5 mM leads to complete deterioration of the alginate within a few hours. Therefore, 3.15 mM was chosen as the maximum level for this experiment:

				C:N:P
1)	*0.5 mM lactate*			(10:1:5)
	1.5 mM C	0.15 mM N	0.75 mM P	
2)	*1.0 mM lactate*			(10:1:5)
	3.0 mM C	0.30 mM N	1.5 mM P	
3)	*5.0 mM lactate*			(10:1:5)
	15 mM C	1.5 mM N	7.5 mM P	
4)	*10 mM lactate*	(0.1X BSM)		(10:1.2:1.1)
	30 mM C	3.75 mM N	3.15 mM P	
5)	*20 mM lactate*	(0.1X BSM)		(100:6.3:5.2)
	60 mM C	3.75 mM N	3.15 mM P	
6)	*30 mM lactate*	(0.1X BSM)		(100:4.1:3.5)
	90 mM C	3.75 mM N	3.15 mM P	
7)	*50 mM lactate*	(0.1X BSM)		(100:2.5:2.1)
	150 mM C	3.75 mM N	3.15 mM P	

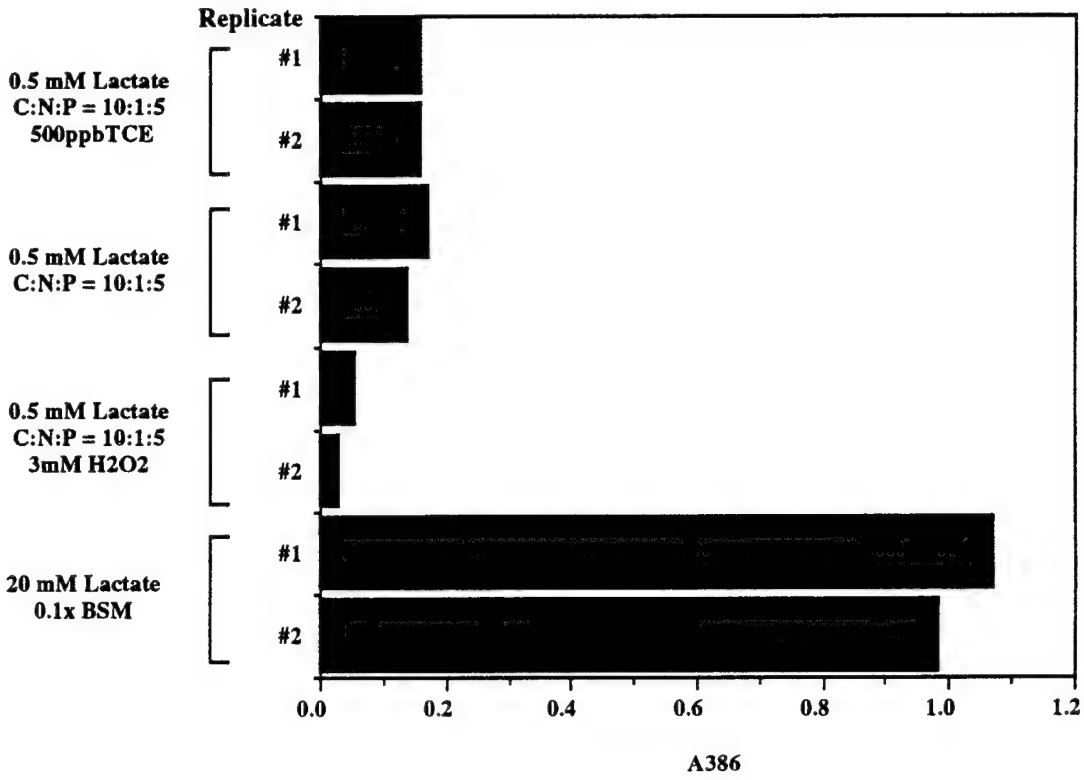


Figure 56. TFHA Production by Alginate-Encapsulated 249 (TOM_{31c})

Based on these data, following a 24 hour exposure, the maximal TFMP oxidative activity was achieved at a moderate lactate concentration of 10 mM, at the C:N:P ratio of 10:1.2:1.1 (Figure 57). Dissolution of the alginate was observed after just a few Days at high carbon concentrations. This may be a result of outgrowth by entrapped cells.

Another shake-flask experiment with alginate entrapped 249 (TOM_{31c}) was run to determine the correct nitrogen and phosphorus concentrations for maximum activity. This experiment was divided into two sets. Both were set to low P concentrations (0.75 mM) in view of the fragility of the alginate polymer, and the competitive effect of phosphate for the calcium cofactor in the alginate matrix to form hydroxyapatite ($CaPO_4$). This experiment was divided into two sets of nutrient ratio effect determinations:

Set 1: was designed to measure the effect of varying C:N&P ratios while retaining the same N&P concentrations and varying only the carbon concentration.

Set 2: was designed to measure the same parameter while retaining the same C:N ratio in face of a fixed P concentration.

	Set 1 (C:N&P)	(C:N:P)	Set 2 (C&N:P)	(C:N:P)
1)	*0.5 mM Lactate*		1)*0.5 mM Lactate*	
	0.15 mM N, 0.75 mM P	(10:1:5)	0.15 mM N, 0.75 mM P	(10:1:5)
2)	*5.0 mM Lactate*		2)*5.0 mM Lactate*	
	0.15 mM N, 0.75 mM P	(100:1:5)	1.5 mM N,0.75 mM P	(100:10:5)
3)	*10 mM Lactate*		3)*10 mM Lactate*	
	0.15 mM N, 0.75 mM P	(200:1:5)	3.0 mM N, 0.75 mM P	(200:20:5)
4)	*20 mM Lactate*		4)*20 mM Lactate*	
	0.15 mM N, 0.75 mM P	(400:1:5)	6.0 mM N, 0.75 mM P	(400:40:5)
5)	*30 mM Lactate*		5)*30 mM Lactate*	
	0.15 mM N, 0.75 mM P	(600:1:5)	9.0 mM N, 0.75 mM P	(600:60:5)

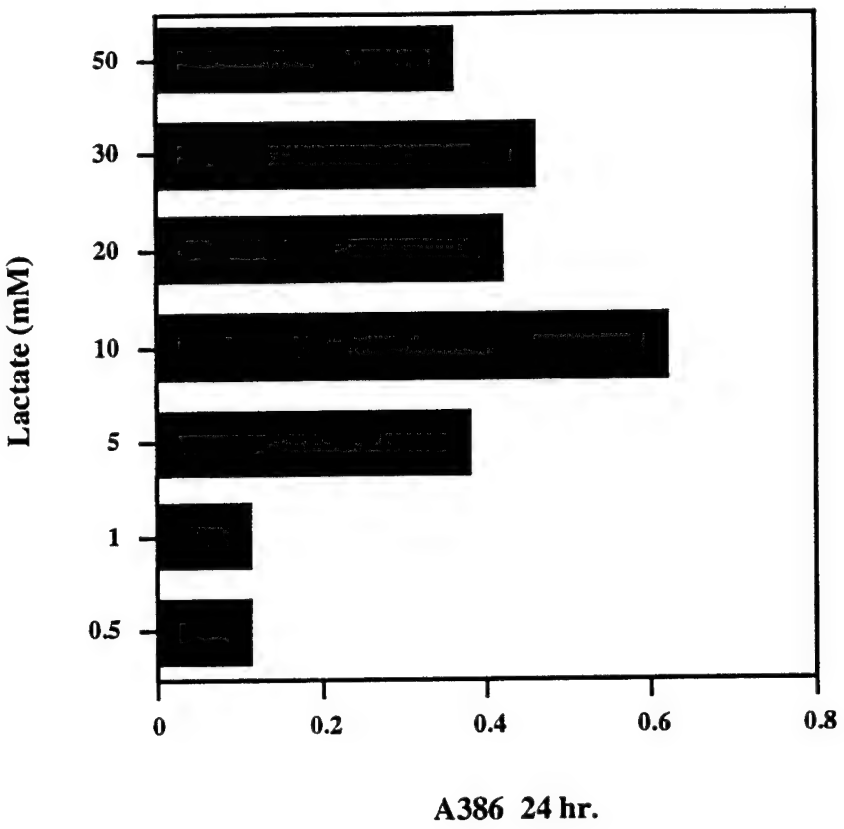


Figure 57. TFHA Production by Alginate-Encapsulated 249 (TOM_{31c}); a Shake Flask Study.

(1). Set 1. C:N ratio effects. The effect of a varying C:N ratio on 249 (TOM_{31c}) was apparent while holding the N&P ratios constant. The maximal activity was related solely to the concentration of the lactate co-reductant (Figure 58), but somewhat surprisingly in an inverse fashion. The lowest carbon concentration (10 mM lactate) corresponding to the lowest C:N:P ratio (10:1:5) gave the highest activity.

(2). Set 2. C:P ratio effects. The effect of varying the C:P ratio on TFMP oxidation rates of 249 (TOM_{31c}) were measured while holding the P concentration constant. The nitrogen was linked to the carbon concentration at the predetermined maximal activity C:N ratio of 10:1 (Set 1 results) . With the exception of the 20:1 C:P ratio there was a marked increase in activity at the lower C:P ratios. Like set 1, the highest activity measurements at 72, and 120 hours were obtained at the lowest lactate concentration (10 mM). In fact, at 24 hours the Tom activity of all the higher lactate concentration amended cultures were higher in set 2, and relatively reversed by 72 hours and remained so by 120 hours.

The alginate beads from the 20 mM and 30 mM lactate flasks of this set dissolved after 3 Days. This seems to support the assumption that growth of the cells may be a contributing factor in the dissolution of the alginate beads.

4. Bioreactor Application of 249 (TOM_{31c})

a. Fluidized Bed Reactor of Alginate Immobilized 249 (TOM_{31c})

A major reason for determining the C:N:P effects on 249 (TOM_{31c}) Tom activity was to test such calcium alginate encapsulated cells in a 1 L fluidized bed reactor. The goal in this reactor study was to attempt a balance between oxygen and lactate concentrations for optimal degradation of TCE.

The reactor was initially filled with alginate beads in 0.5 M $CaCl_2$. The liquid was recycled from top to bottom at 1700 mL/min. This recycle rate was sufficient to maintain the alginate beads (~2 mm) in a fluidized bed. The carbon source was added at 2.8 mL/min. The TCE was added at 1.5 mL/min and pure oxygen at 2.8 mL/min. The column was first run for 24 hours with just carbon and oxygen. On Day 2, TCE was started. On Day 3, samples were taken from the influent and effluent ports for TCE determinations. A gas sample from the effluent port was also taken to monitor TCE loss due to air stripping.

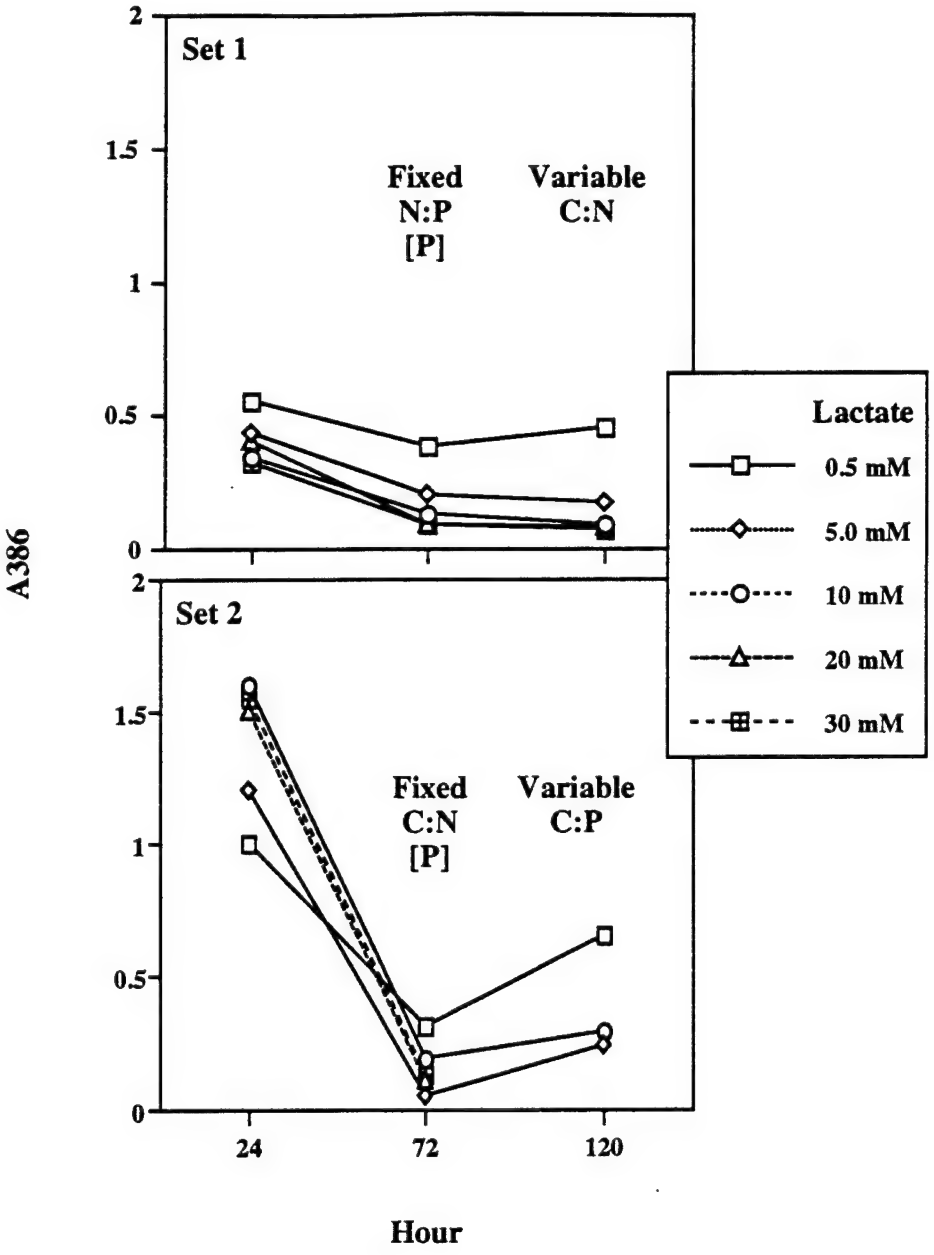


Figure 58. C:N and C:P Ratio Effects on the TFMP Oxidative Activity of 249 (TOM_{31c}); Shake Flask Studies

The column was run in this fashion for 3 Days. On Day 6, the column was poisoned with sodium azide at 10 mg/mL. TCE monitoring was continued for another 3 Days and the experiment was terminated.

Feed stock	Concentration after dilution
15.3 mM Lactate	9.9 mM Lactate
0.45 mM N	0.29 mM N
1.0 mM P	0.65 mM P
1.0 mM $CaCl_2$	0.65 mM $CaCl_2$
0.1 X Trace Metals	0.065 X Trace Metals

The column showed an average TCE loss of 79% attributable to degradation (Figure 59) with a 20-30% loss to air stripping (as determined by the azide poisoned control) .

While this rate of TCE degradation was promising and probably even useful in an application sense, the maintenance of the alginate polymer was not practical, even in the presence of 1.0 mM $CaCl_2$.

b. Bio-Ox^{tm} Reactor Application of 249 (TOM_{31c})

In June of 1994 we began testing SRE Inc.'s Bio-Ox^{tm} reactor with the strain 249 (TOM_{31c}) using phthalate as a carbon source. A plan view of the reactor design is given in Figure 60. Cells were grown up and resuspended in a 0.5xBSM solution to an absorbance density of 0.97 at 600nm. This was then recirculated in the reactor according to the side view operating diagram in Figure 61, for 46 hours until the bacterial density was reduced 0.27. According to the manufacturer this represented cells lost from solution due because they become imbedded in the coiled membrane. The recirculating cells remained TFMP positive throughout this immobilization phase. On Day 3 a 0.5mM phthalate, 0.1xBSM solution was begun at 3mL/min flow rate. From this point until the end of this test the carbon concentration was varied from 0.01mM C to 1.75mM C while monitoring its effects on the dissolved oxygen in the recycle line of the reactor. At that point TCE at a concentration of 400 µg/L was begun at 3mL/min with the inputs and outputs measured daily (see Figure 62). No degradation of TCE was apparent.

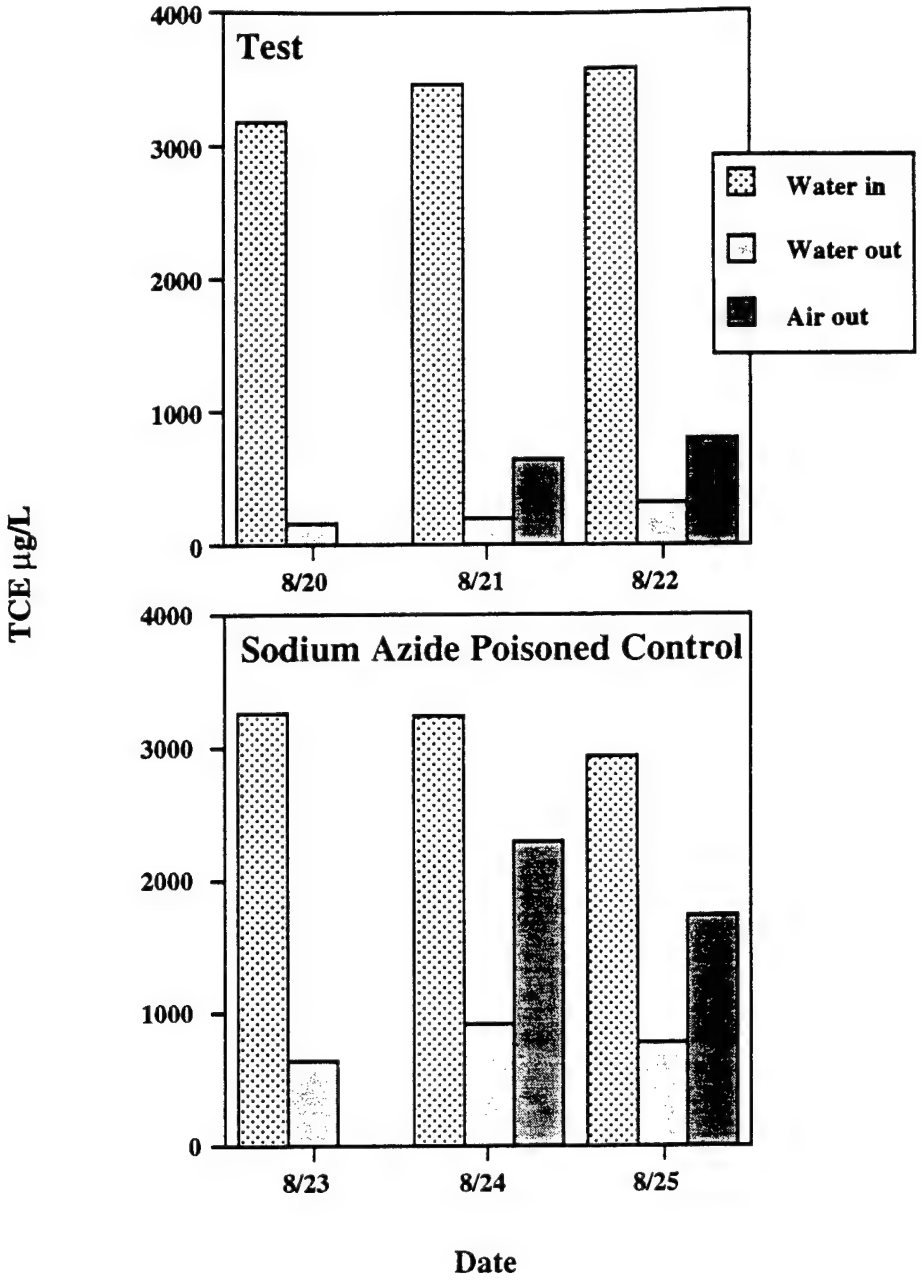


Figure 59. TCE Mass Balance Through a 1 -Liter Fluidized -Bed Reactor Containing Alginate Encapsulated 249 (TOM_{31c}) Before and After Poisoning with Sodium Azide

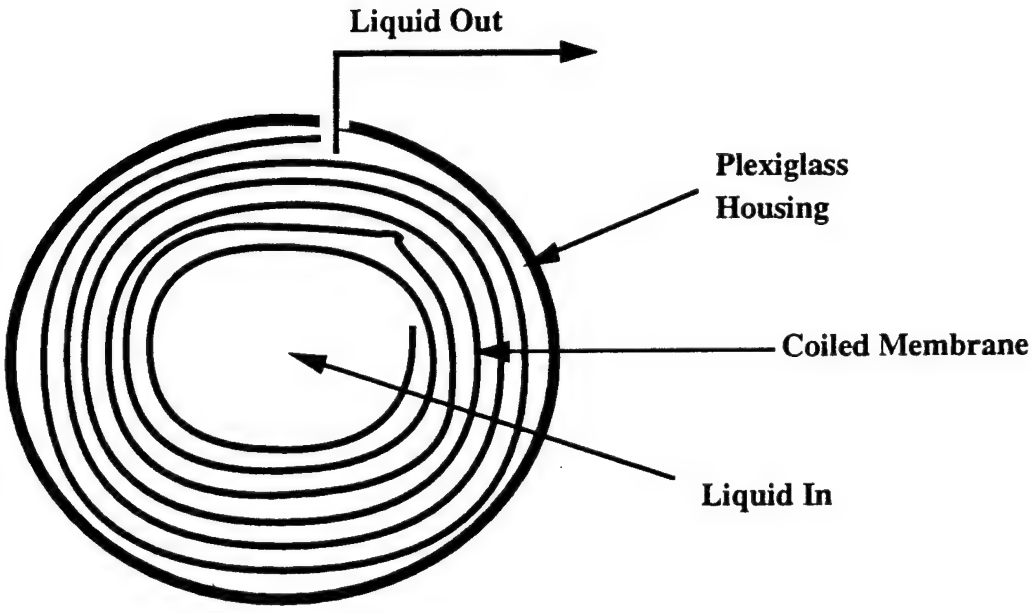


Figure 60. Plan View of the Bio-Ox Spiral Membrane Bioreactor

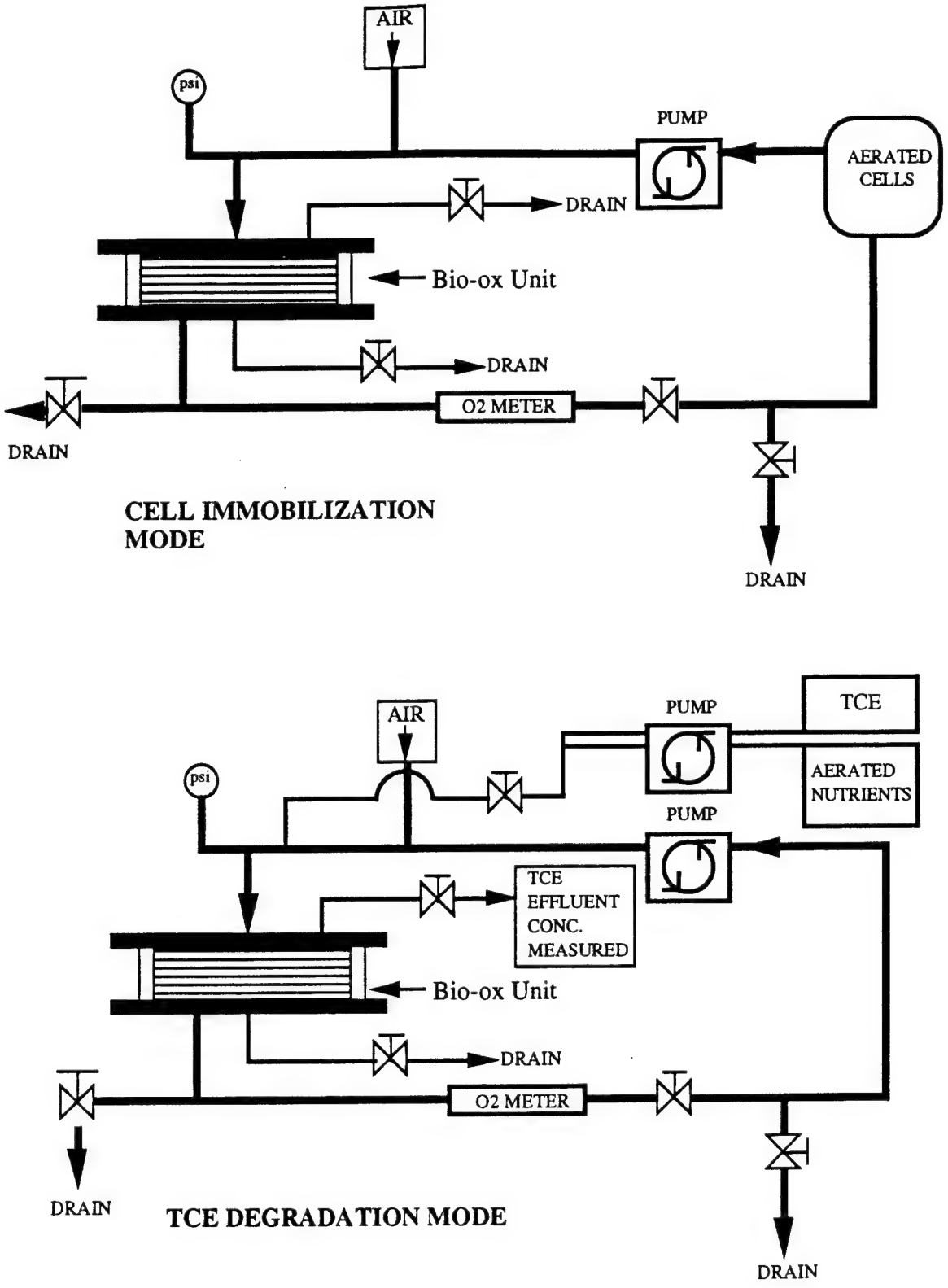


Figure 61. Operational Diagram of the Bio-Ox Spiral Membrane Reactor

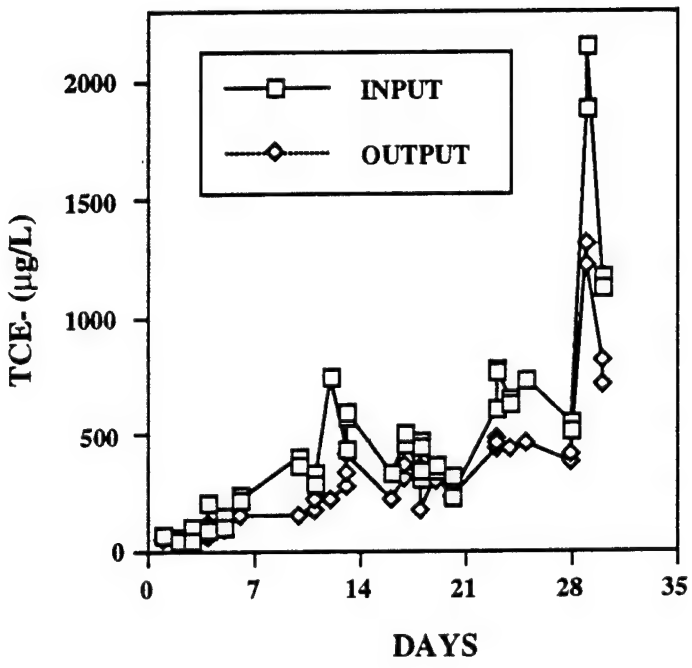


Figure 62. TCE loss in the Bench Scale Bio-Ox Spiral Membrane Reactor

The reactor was tested on Day 12 to determine if respiring cells were still present. An oxygen uptake experiment was done in which all inputs and outputs were sealed and the oxygen concentration monitored. This indicated an oxygen demand of 1.9 mg O_2/hour.

During the conclusion of this test, two abiotic controls were utilized for three Days each: 1) a nitrogen purged feed stock resulting effectively lowered the dissolved oxygen in the reactor to levels where the oxygenase is known to be incapable of TCE oxidation. Under these conditions any loss of TCE must be considered abiotic, and 2) The reactor was sterilized with 0.5M HCL, killing and removing the cells. The nitrogen test resulted in an average TCE loss of 35% (std. dev. = 4.7%) and the sterilized reactor averaged 32% loss (std. dev. =7.4%). This compared with the degradation test average of 35% (std.dev. =18%). From this it was concluded that the presence of organisms had little effect on the loss of TCE in this reactor system. This was in spite of the fact that at Day 35 a micro test reveled 7% of the effluent cells were TFMP positive at $1.95x10^5$ TVC. It would appear that the reactor itself (membrane, epoxy resins or plexiglass housing) absorbs and releases TCE according to the concentrations to which it is exposed (see Figure 63). This was true even at higher input concentrations experienced at the end of this test.

At the end of July, 1994, a second test was begun using the 249 (TOM_{31c}) strain but utilizing 0.25mM lactate as a carbon source. The initial immobilization was in 2 phases. First a 3.6 absorbance (at 600 nm) cell culture in 0.5xBSM was reduced to 2.6 absorbance in 26 hours. Two Days later, remaining cells were reinoculated in the reactor resulting in a change of absorbance from 3.2 to 1.6. The lactate and the TCE at 800µg/L was used at the same flow rates as the above test for the following eight Days. The results of this test were a 25% TCE loss with a standard deviation of 13%.

At Day ten we decided to change the reactor into a CSTR to find out what the optimal TCE removal rate was in this reactor. This resulted in only a 22% loss with a standard deviation of 9.6%.

Water saturated with TCE was recirculated through the reactor while measuring the input and output concentrations. It was found that the membrane of the reactor took five Days to reach the point where 10% of the input TCE was still unaccounted for, and presumed absorbed. Since the water saturated concentration of TCE (~8mM) were approximately 100 times higher than the environmentally relevant concentrations needed to test the reactor (~ 10 µM) it was concluded that this reactor design was not appropriate.

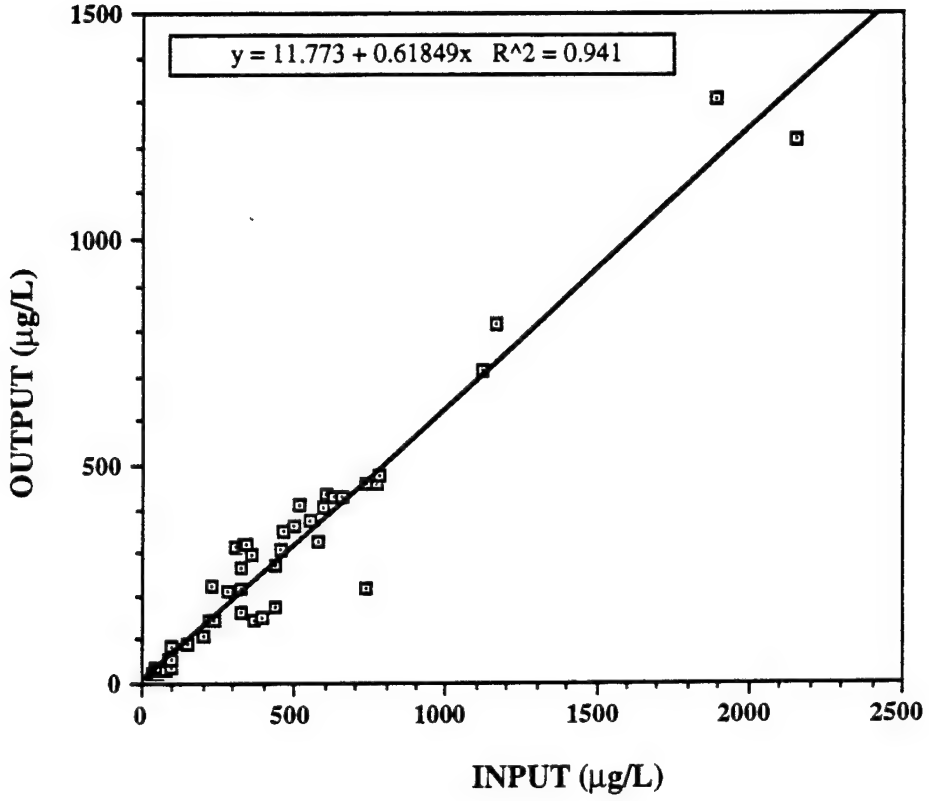


Figure 63. TCE Input & Output from an Abiotic Bio-Ox Spiral Bioreactor

SECTION IV
IN SITU APPLICATION OF $PR1_{31}$
AND
SELECTED TOM_{31c} TRANSCONJUGANTS
AT NAS WHITING FIELD

A. INTRODUCTION

The final phase of this investigation was to test the *in situ* application of the constitutive TCE degraders developed thus far in this project. This included the strains: $PR1_{31}$ (TOM_{31c}), JS150 (TOM_{31c}), 249 (TOM_{31c}). Since this study coincided with the development of new TCE degradative strains through another Air Force sponsored study, another transconjugant strain was incorporated into this study: WS-23 (TOM_{31c}). This strain was isolated from an aquifer sample of the Gilbert Mosely Superfund Site, Wichita KS (provided courtesy of Dr. Al Bourquin). The immediate goal was to test the effect of various chemical amendments on the degradative capacity of these bacteria in field material. It had already been observed under lab conditions that there was a substantial requirement for nitrogen (i.e. more so than carbon or phosphorus). This might reflect a need for nitrogen in the turn over the degradative enzyme to maintain effective degradation rates. This would certainly be in keeping with the theory that these enzymes are inactivated by oxygenated reactive intermediates, but simple enzyme activity decay based on the half-life of catalysis could also explain this.

Experience to this point led us to believe that a static system requiring a stable biofilm or immobilized cells was not practical. An *in situ* groundwater recirculation well was chosen as the method of *in situ* treatment to model for eventual field application. Two major reasons for this decision were the relative availability of this technology, and its ease of modeling in above ground columns. This could be provided for field scale application in the event of successful test results, by several commercial systems currently available. The goal was therefore to model in untreated saturated soil columns (in an above ground test) the effects of adding constitutive TCE degrading strains of bacteria, to such an *in situ* recirculator, and to monitor the progress of pollutant removal relative to a similarly operated control unamended with laboratory developed bacteria.

An *in situ* recirculation system (modeled after the situation depicted in Figure 64) was designed. It was intended to mimic an area representing a core of the area affected by recycle in the vadose zone or aquifer (depending on the depth of the recycle outlet screens) immediately beyond

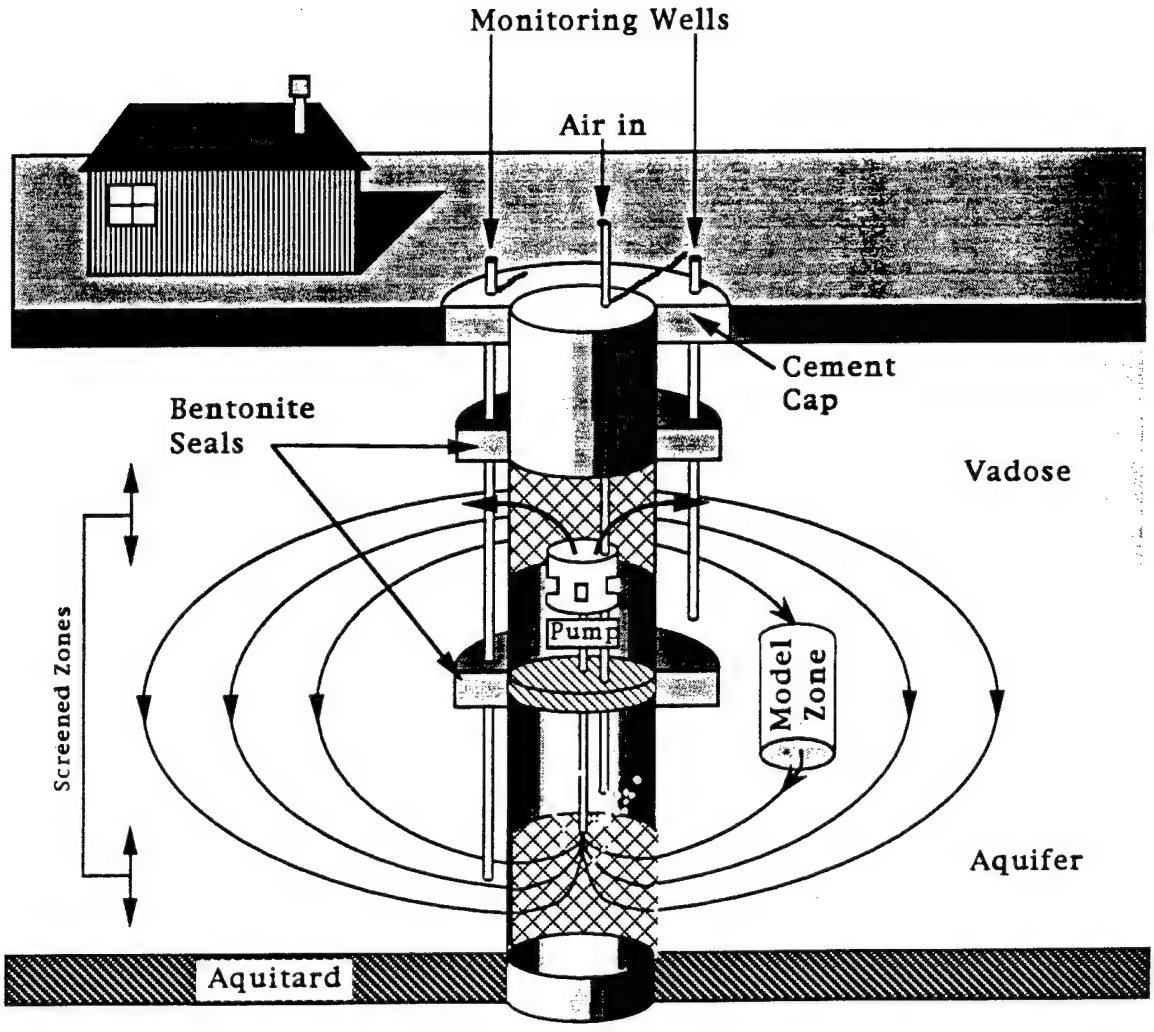


Figure 64. Subsurface Groundwater Recirculation System

the upper screen of the in situ recycle well. This region will be the first to encounter the bacteria displaced from this system as well as the pollutants recycled through the treatment zone. In this way we hoped to recreate an effective model of the immediate treatment zone surrounding the well.

By establishing a high input level of oxygen, and minimal levels of nutrients in the regions surrounding the in situ circulation well, one should have adequate conditions for the constitutive degradation of TCE by these constitutive bacteria. Based on the results in both our lab and the Boston field test of the bioreactor, it would seem that the physical conditions are not necessarily as limiting to this process as the biological variables. This is seen as primarily due to :

1. Competition for the necessary nutrients by indigenous, highly competitive bacteria, and

2. Survival in the face of predation. Both factors will combine to determine the stable upper level bacterial numbers achievable for our introduced bacteria.

Since we already know the lower threshold of effective treatment will be a fairly high concentration, i.e. ~10^7 cfu/g (Krumme Timmis and Dwyer, 1993), these factors become extremely important. Because we need to maintain such high population densities this means that our selected strains must be maintained at levels that would ordinarily not be stable. These population levels would mean a radical conversion of the extant bacterial population to one in which our organism is the most common. Since normal population densities of these sediments rarely exceed 10^8 bacteria per gram, this means we want to establish and <u>maintain</u> a situation in which at lease one in every ten bacteria is one of our degraders. Ideally we would want to push these populations to 10^9 per gram where our degraders would outnumber the native bacteria by ten to one. It is very difficult to maintain such a situation. Natural controls soon assert themselves. The more populous bacteria are by default more often targets of predation. Also, the capacity to maintain these differences merely by selective feeding of these strains is also unlikely as the carbon sources utilized by these strains are also easily utilized by indigenous bacteria.

We therefore added nutrients to the system to allow for both enzyme turnover as well as cell replacement. These additions will occur sequentially, and the model systems (i.e. columns) monitored continuously for:

1. The physical parameters: temperature and dissolved oxygen (DO);

2. Chemical makeup: GC analysis of the input and output streams; and

3. Bacteriological enumeration of the total heterotrophic population (capable of growth on R2A (see below)) and the phenol-utilizing, kanamycin-resistant population (of which all our constructed strains will belong).

Since the mobility of these strains in Whiting Field subsurface material is not known, these populations will be monitored throughout the study from column effluent water, and at the termination of the experiment from the column material itself.

B. MATERIALS AND METHODS

1. Organisms and Culture Conditions

WS23 (TOM_{31c}) was produced through conjugation and selection as described in section III for JS150 (TOM_{31c}). Other bacterial strains are previously described.

2. Description of the Test Site

Because the Whiting Field site is designated an NPL site it is under Superfund jurisdiction. As such the ensuing regulations precluded the possibility of locating the field laboratory directly at one of the contaminated wells. For this reason, it was decided to pump contaminated site water to barrels and transport to the nearest location. This was provided for us by Whiting Public Works personnel. Whiting field (Figure 65) is located approximately 20 miles east of the University of West Florida, making it convenient as a field test site. The TCE plume in South Field that originates from the wash rack area seen in Figure 66 was chosen as the material for our tests. The lab and associated columns were located approximately 900 feet North of wells WHF 1466-13 and 30-3, and about 300 feet from well 7-1. It was not possible to obtain more than 50 gallons of contaminated water from any one monitoring well. For this reason it was decided to perform the experiment in three phases:

PHASE I Contaminated water from WHF 1466-13, amended with carbon, nitrogen and phosphorus.

PHASE II Contaminated water from WHF 30-3, amended with nitrogen and phosphorus.

PHASE III Contaminated water from WHF 7-1, amended with carbon.

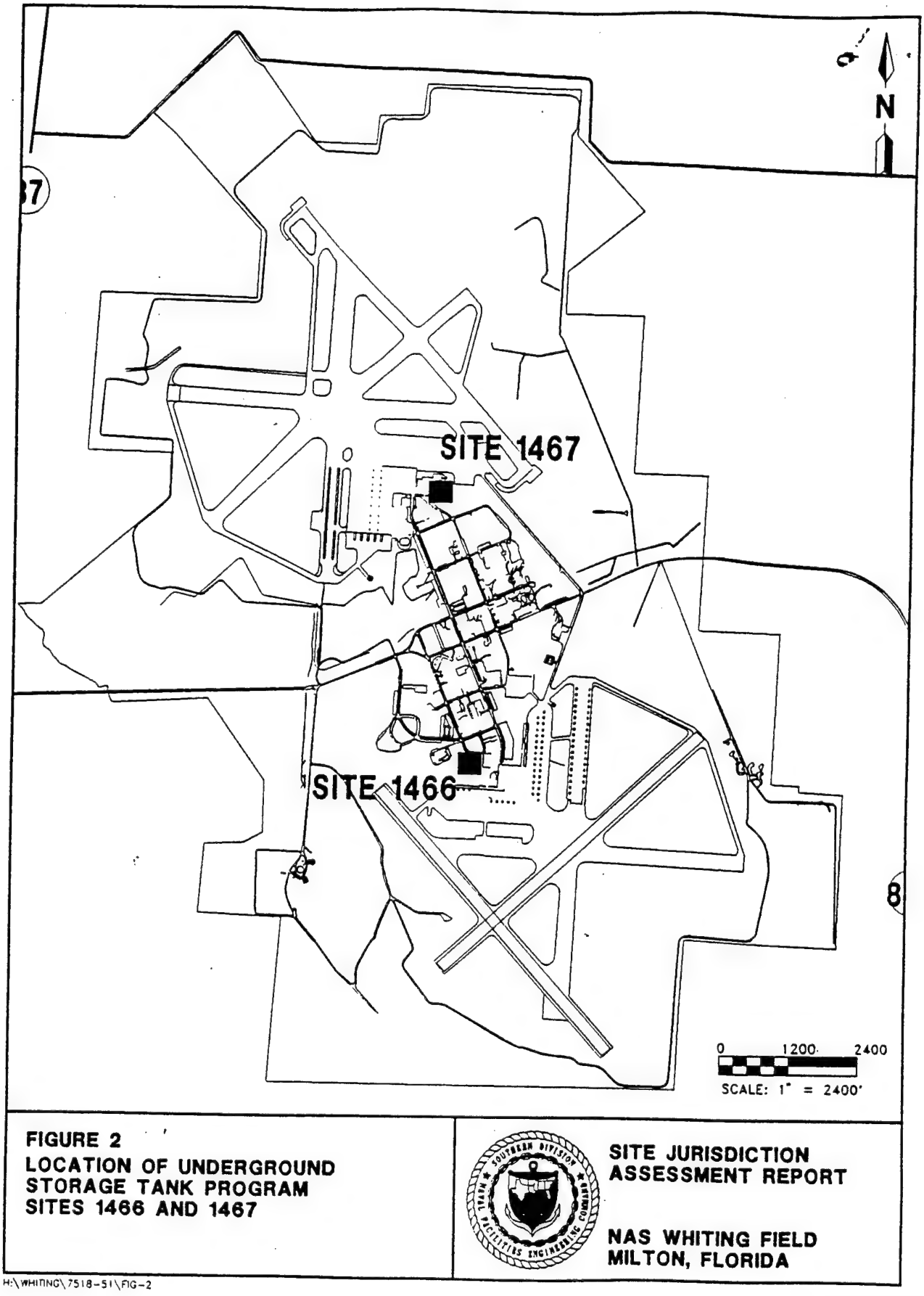


Figure 65. NAS Whiting Field. Large Scale Map.

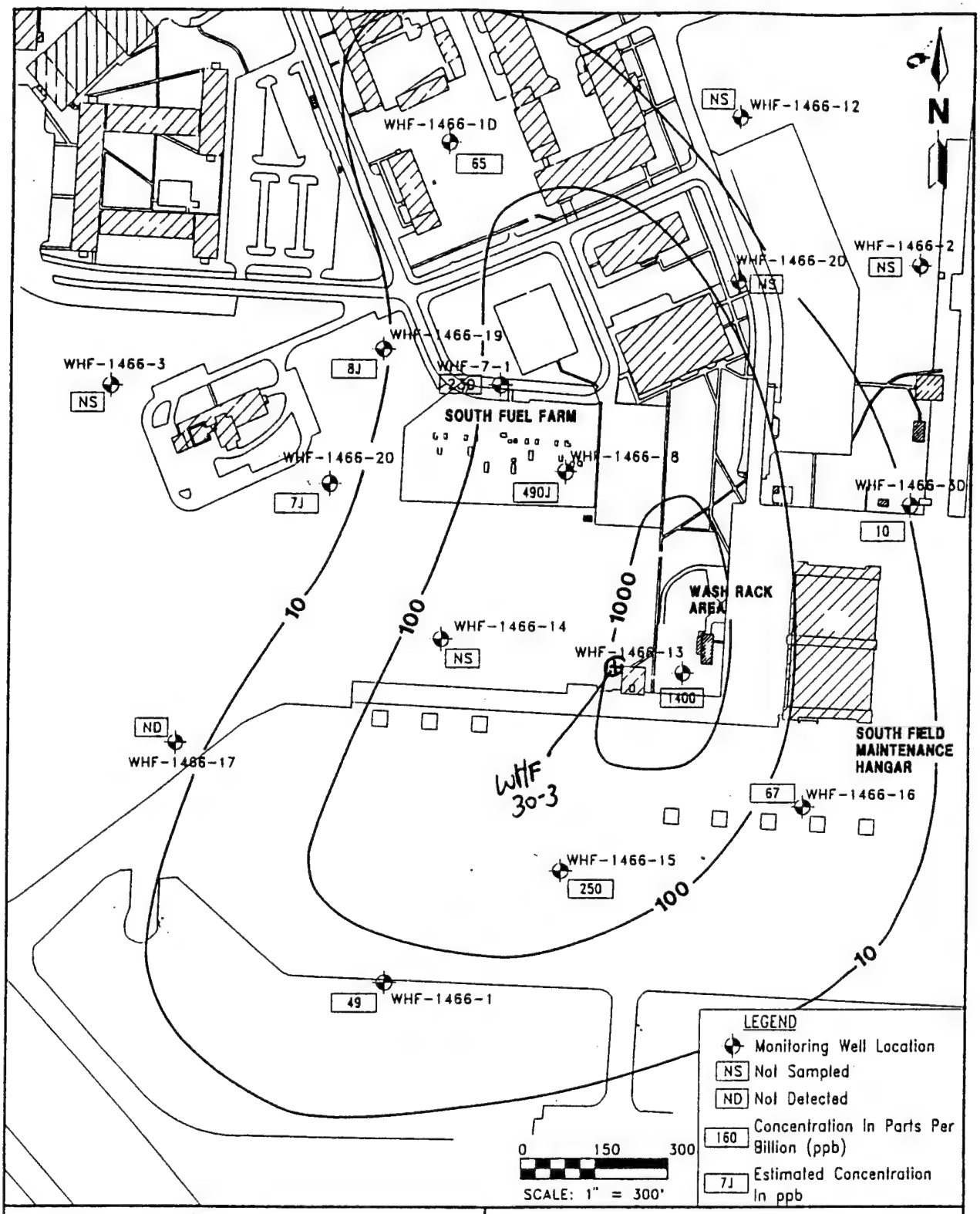


FIGURE 10
TRICHLOROETHENE ISOCONCENTRATION MAP,
UST PROGRAM SITE 1466,
IR PROGRAM SITE 7

SITE JURISDICTION ASSESSMENT REPORT

NAS WHITING FIELD
MILTON, FLORIDA

H:\WHITING\7518-51\FIG-12

Figure 66. Site Scale Map of NAS Whiting Field: South Field.

Because the materials were taken from three sites, large variation in contaminants were anticipated. Wells 1466-13 and 30-3 were expected to have a high TCE concentration with barely detectable BTEX contamination based on the most recent site assessment information available. Well 7-1 was expected to have the highest BTEX levels and the lowest TCE.

3. Above Ground Model Design Considerations

The test columns were designed to mimic anticipated flows that would be present in a lateral core of material (aquifer or vadose), immediately adjacent to the output screen of an in situ recycling well in which bioaugmentation was taking place. They were constructed of glass process pipe and packed with subsurface soil collected below three feet from a wooded section in North Field. Pipe of two sizes: 6 inch, and a 2 inch internal diameters (ID) were used in their construction. The four larger were used for the control and for strains *P. cepacia* $PR1_{31}$ (TOM_{31c}), *P.* sp JS150 (TOM_{31c}), and *P. cepacia* 249 (ATCC # 17616) (TOM_{31c}) . One of the two smaller columns received BR-23 (TOM_{31c}) and lactate; and the other 5% ethanol.

4. Details of Construction

A diagram of the column is seen in Figure 67. It consists of the 6 inch ID glass column described for the earlier bioreactor work (phases I and II). This design was used for strains and the uninoculated negative control. Two smaller ones were used for the new strains: , and were identical to the 6 inch columns in construction. Nutrient delivery was by means of peristaltic pumps delivering either a nutrient concentrate of 50% BSM, or water (depending on the treatment phase). Each large column outlet was equipped with quick disconnect fittings that allowed the transient routing of column effluent through a flow-through oxygen probe (Nester type). A schematic illustrating the arrangement of all columns, pumps and water influent flow routes is given in Figure 68.

Oxygen was added to the column by means of the bubbleless transfer of oxygen saturated water as described in phase II.

5. Details of Operation

a. Oxygen Addition

Oxygen in the barrelled site water measured $\leq$ 2.5 mg/L. Laboratory tests indicated an absolute need for O2 $\geq$ 2.0 mg/L for enzyme function. Oxygen addition to the columns was afforded by means of an oxygen saturated water stream as described in phase II. Column effluents were periodically measured for oxygen content.

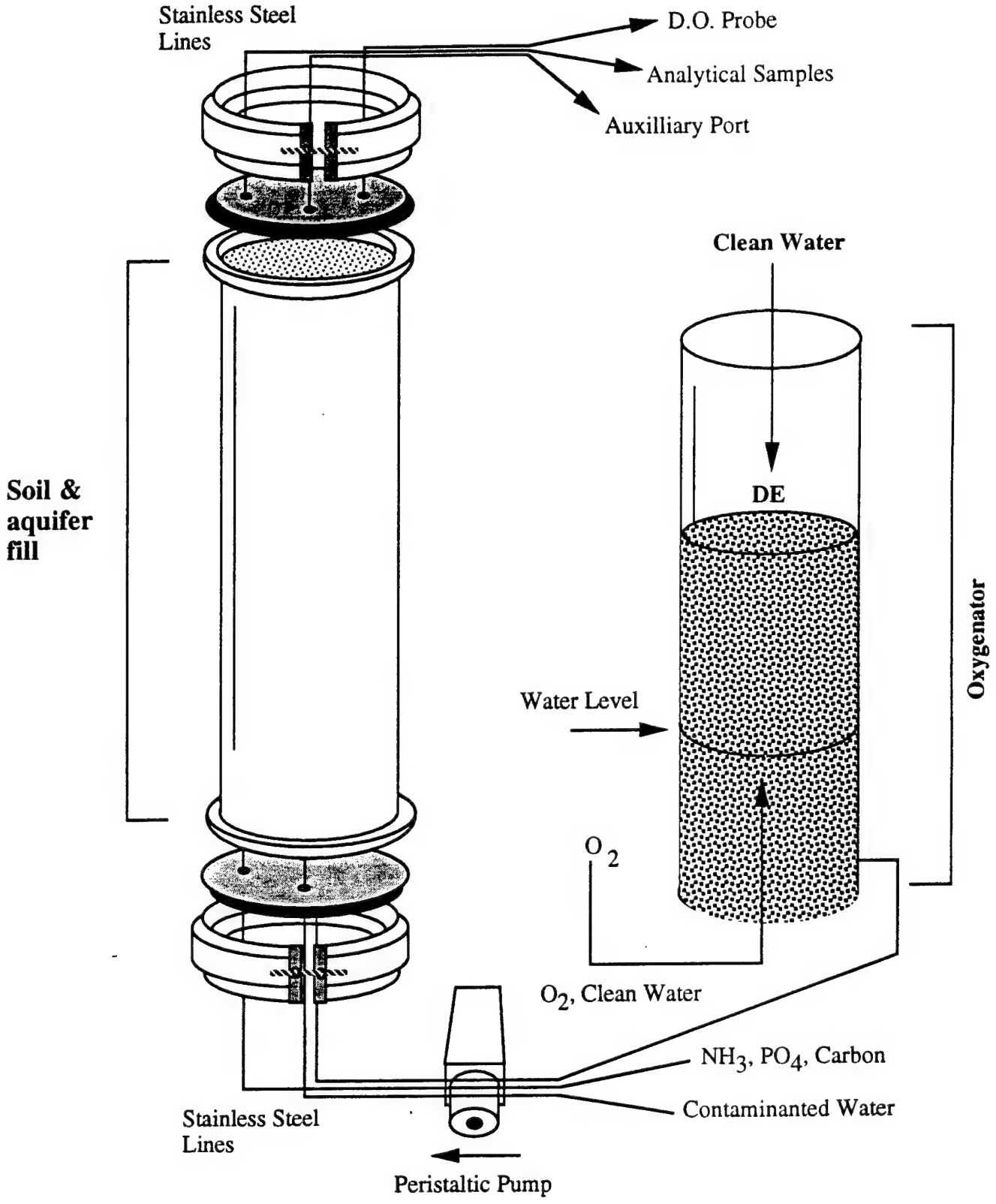


Figure 67. Diagram of 22-Liter Reactor as Used for the Field Test at NAS Whiting FilELd

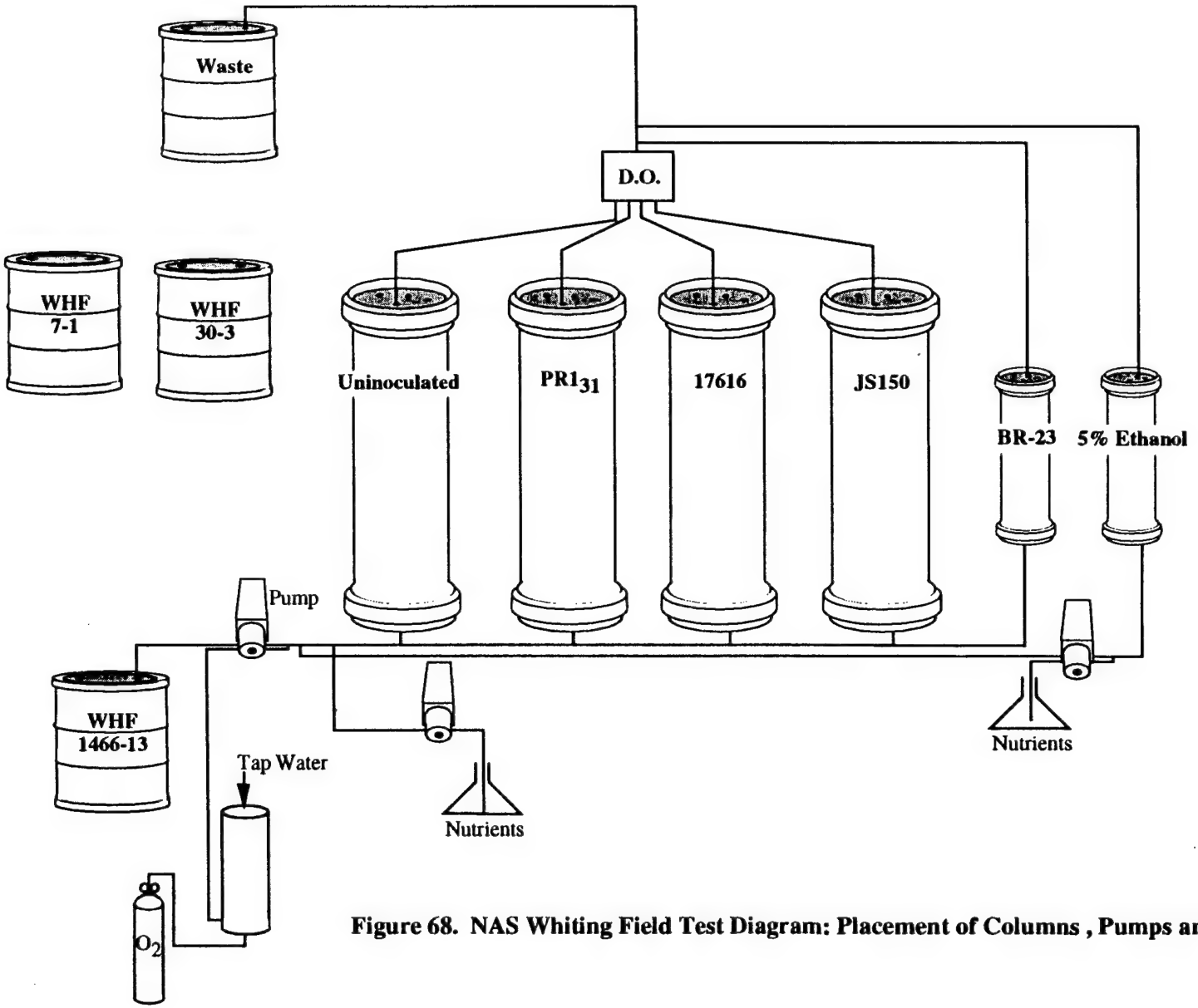


Figure 68. NAS Whiting Field Test Diagram: Placement of Columns , Pumps and Barrels

b. Temperature Monitoring

Because of the placement of the mobile lab the columns were mounted on the west side. This resulted in their receiving afternoon sun. Because of this daily warming and cooling cycle we installed hi/low thermocouples on each of the large columns to record these temperature fluctuations.

c. Rate of Contaminated Water Addition

Contaminated site water was pumped from the holding barrels to each column in an ascending direction. A filled column was drained and the void volume was determined to be 5500 ml. The water flow rate was calculated to represent two volume changes per day that should be attainable in the zone of influence of an in situ recirculation well:

$$11{,}000 \text{ ml}/[(24 \text{ hr})(60 \text{ min/hr})] = \underline{7.64 \text{ ml/min}}$$

(1). Phase I: WHF 1466-13: Addition of carbon (lactate), nitrogen (ammonia) and phosphorus (phosphate).

Phase I included both organic (lactate) and inorganic (nitrogen and phosphorus) addition. Well water WHF 1466-13 was selected for this phase. 7.0 mg/L assumed to be a minimal oxygen level attainable in the column water. To balance the maximal flow rate of carbon to the column the following calculations were used:

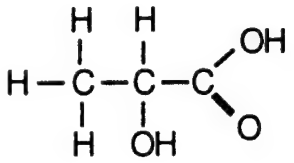
(7.64 ml/min) (7 µg O_2/ml) = 53 µg O_2 delivered per minute

(53 µg)/(32 µg/µmole) = 1.66 µmoles O_2/ min

(1.66 x 10^{-6} moles O_2/min) / (7.64 x 10^{-3} liters water/min) = 2.17 x 10^{-4} moles/l

minimal input water [O_2] = 2.17 x 10^{-4} M O_2

This would be sufficient to completely oxidize one third as much lactate,



according to the equation:

$$C_3H_6O_3 + 3O_2 \longrightarrow 3CO_2 + 3H_2O$$

or:

$(2.17 \times 10^{-4}\ M)\ (0.33) = 7.23 \times 10^{-5}\ M$
$= 0.1$ mM lactate

Note: this mass balance is valid only as a guide since it is calculated as if all the lactate were consumed during passage through the column, and there was no additional oxygen demand due to contaminants in the groundwater or the added bacteria.

The feed barrel containing well 1466-13 water was then amended to 0.1 mM lactate and 0.5 x BSM, and sparged with pure oxygen for 2 minutes, and sealed. Nitrogen (NH_4^+) and phosphate (PO_4^{-2}) (supplied as BSM) were present at final concentrations of $K_2HPO_4 \cdot 3H_2O$, $NaH_2PO_4 \cdot H_2O$, NH_4Cl 2.12, 0.5, and 1.0 g/l respectively. The level of contaminants in the feed barrel began to fall following these additions, presumably due to the activity of the native bacteria. Because of this the other contaminated site waters were not amended with oxygen, carbon, nitrogen, or phosphorus prior to their introduction to the test columns. BR-23(T) was not introduced until phase II.

(2). Phase II: WHF 30-3: Addition of nitrogen (ammonia) and phosphorus (phosphate)

Phase II represented only inorganic additions of nitrogen and phosphorus (primarily). WHF 30-3 water was selected for this phase. Inorganic amendment was accomplished by adding BSM to the 30-3 barrel to a final concentration of 0.5 x as in phase I.

(3). Phase III: WHF 7-1: Addition of carbon (lactate)

Like phase I, lactate was added to the feed barrel to a final concentration of 0.1 mM. Unlike phase I, no BSM or other source of nitrogen or phosphorus was added. WHF 7-1 well water was selected for this phase.

6. Bacterial Culture and Inoculation of Test Columns.

$PR1_{31}$ (TOM_{31c}), 17616 (TOM_{31c}) and JS150 (TOM_{31c}) to be used in the columns were grown first as 5 ml overnight starter cultures in R2A medium. These were in turn used to inoculate 1 liter R2A cultures and grown overnight. Pellets of these cultures, resuspended in BSM were added to each of the large test columns at the following concentrations: $PR1_{31}$ (TOM_{31c}) A_{600} = 2.2; 17616 (TOM_{31c}) A_{600} = 0.75 and JS150 (TOM_{31c}) A_{600} = 2.7 on October 23. A similar reinoculation was performed on November 7 with pellets resuspended from 0.5 liter 17616 (TOM_{31c}) and JS150 (TOM_{31c}) and 1 liter $PR1_{31}$ (TOM_{31c}) overnight cultures.

7. Microbiological Monitoring

Samples of column effluents were routinely monitored for bacterial counts of total viable cells (TVC) throughout the study. These TVC measurements were performed with two media. One media was for enumeration of the total heterotrophic population capable of growth on unsupplemented R2A agar. The other was for the population of phenol utilizers capable of growth in the presence of kanamycin sulfate at 50 μg/ml (km) on phenol at 2 mM as the sole carbon source in purified agar plates containing 1 x BSM. This latter population should include $PR1_{31}$ (TOM_{31c}), 17616 (TOM_{31c}) and JS150 (TOM_{31c}), but would by no means be limited to them.

Since the columns were tested in a upflow mode, and we really did not know what to expect in terms of organism mobility through the Whiting soil matrix, the column soil packings were sampled at the end of the experiment by draining the column and sectioning a soil core through the center. The sections were taken from the uppermost 3 cm, lowest 3 cm and center 3 cm bands of column material. These were collected, aseptically homogenized, diluted and plated to the R2A and Phenol-Km media. The phenol-Km countable plates were pulled to nylon reinforced nitrocellulose discs for colony hybridization to *tom*A gene probes (Shields et al. 1995).

8. Gene Probes

We could not selectively grow just the bacteria added to these columns. Traditional bacterial population monitoring only enumerated the general population to which these organisms belong: i.e. Km resistant phenol degraders. Since their introduction might not significantly alter these numbers (particularly if such populations are numerous to begin with), a method was needed to confirm the presence of the added bacteria at the end of the study before any degradative activity could be ascribed to them and not just the native population.

Three probes were utilized. The *tom* genes were previously cloned by us (Shields et al. 1995) as an 11 kb *Eco*RI fragment. This DNA fragment contains all subunits of the *tom*A gene (six open reading frames evident), the C23O gene and the amino terminus of the hydrolase gene and constituted probe I. Probe II used consisted of the second and third open reading frames of *tom*A: *tom*A1 and *tom*A2, subcloned from this gene region as a single *Bam*HI fragment. Probe III consisted of the 2784 bp internal *Bgl*II fragment of Tn*5*. The stringency of hybridization was controlled by washing at 65°C, in 2x or 0.5x SSC and room temperature at 0.1x SSC (both containing 1% SDS). The maps of all three probes are found in Figure 69.

9. Analytical Procedures

TCE, toluene, benzene, and dichloroethylenes (both *cis* and *trans*)were measured from aqueous samples through purge and trap in line with a Hewlett-Packard 6990 series II gas chromatograph. The GC was equipped with a Vocol capillary column and a photo ionization detector operated at 200 °C. The samples were run with helium carrier (Aux F) 40 psi. The programmed run consisted of the conditions: Oven = 40 °C; Initial temp = 40 °C; Initial time = 4.00; Rate = 5 deg/min; Final temp = 50 °C; Final time = 5.00 min. The injector temperature was 300 °C.

The retention times recorded for the four analytes were: *cis* and *trans*-DCE, 1.4 min; Benzene, 2.0 min; TCE, 2.4 min; and toluene, 3.6 min. The *cis*- and *trans*- isomers of DCE were not separable under these conditions, and were consequently reported as the total mixture concentration.

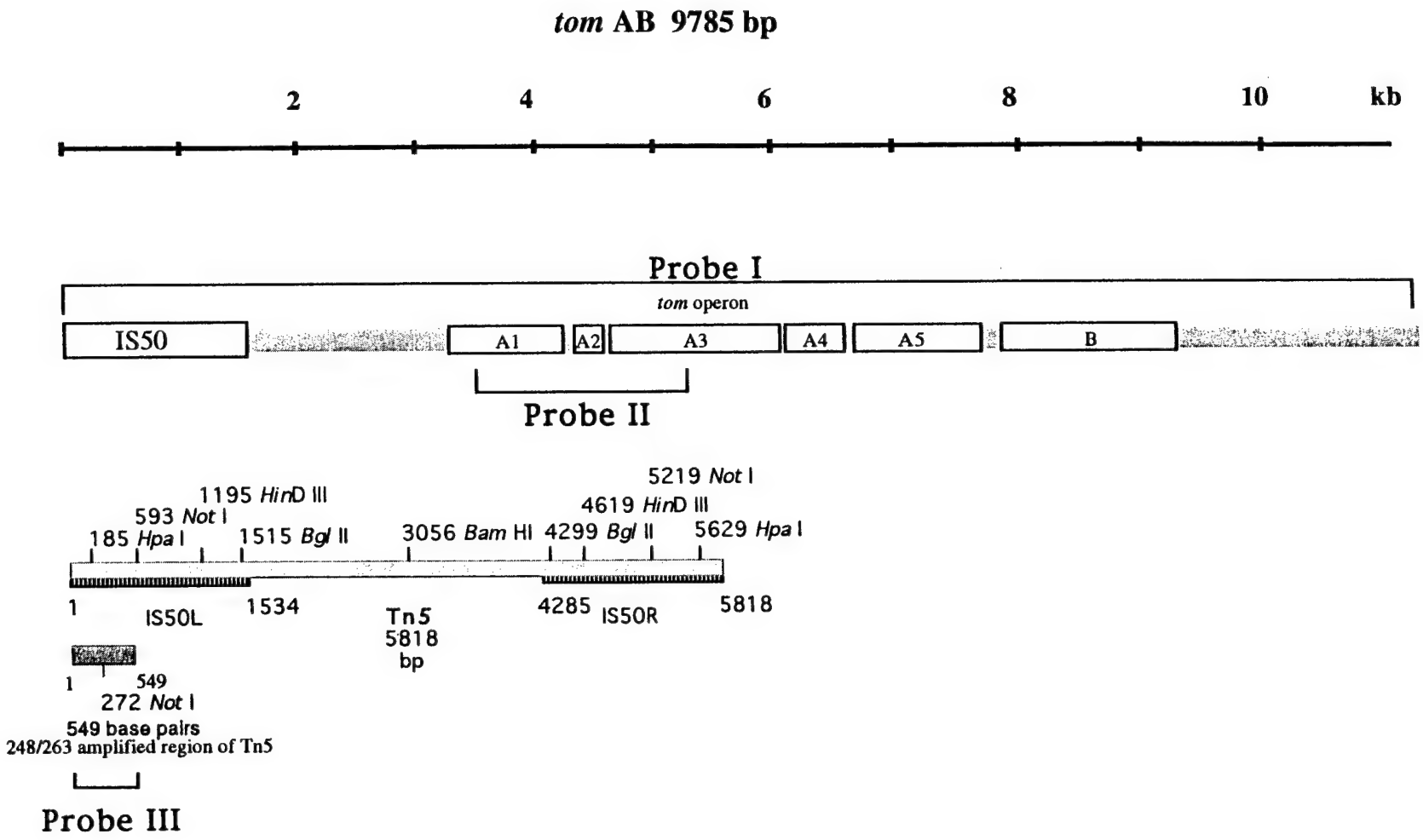


Figure 69. DNA Probes I, II, and III.

C. RESULTS

1. Inoculation of Soil Columns

Preliminary data in both our lab and others (Krumme, Timmis and Dwyer, 1993) demonstrated a minimal cellular concentration to achieve significant TCE degradation rates by the constitutive $PR1_{31}$ Our initial inoculation of bacteria to the field material was therefore to 10^7 cfu/g, a density calculated to be the minimum requirement to achieve effective metabolism of TCE. The use of carbon, phosphorus and nitrogen amendments, and selected site water sources are given in the calendar of events shown in Figure 70.

2. Oxygenation of contaminated site water materials

The oxygen content of the site water was measured at $\leq$ 2.5 mg/L. Previous studies by us indicated a minimum of 2 mg oxygen/l in order for oxidation of toluene or co-oxidation of TCE by Tom. To increase this oxygen content to a minimum of 7-8 mg/L two methods were tested.

a. Direct addition of pure oxygen to contaminated water from the site with a minimum of stripping. This would be the model most closely fitting the anticipated use of an in situ recirculation well in which oxygen was added through a sparger. The use of such a mechanism would undoubtedly be simpler for overall field construction, but would carry with it the added burden of the need to treat the produced air stream from the well containing the more volatile components.

Addition of pure oxygen to the site materials resulted in the rapid oxidation of residual BTEX components. The presence of such components is a probable indication of oxygen limitations in the site to begin with. Since no particular precautions were taken to collect the 55 gal barrels of site water, we were somewhat surprised to find oxygen levels at only ~2 mg/L. This pointed to the likelihood of a significant BOD already present in these materials. Initial attempts to add pure O2 to the first test barrel resulted in the rapid degradation of the BTEX components over the week long course of its introduction to the test columns.

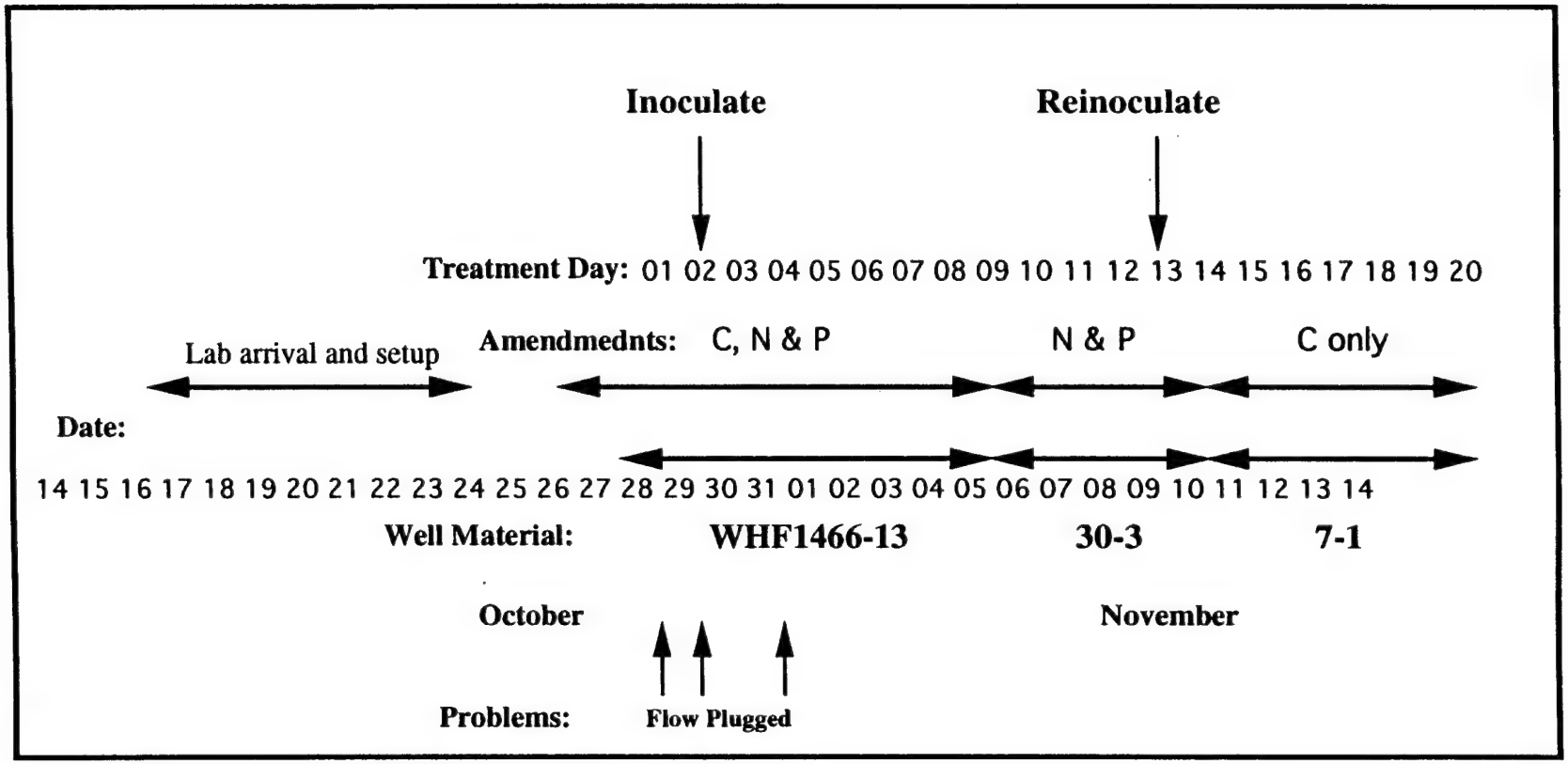


Figure 70. NAS Whiting Field Timetable of Events.

b. Oxygenation of uncontaminated water and mixing this with contaminated site water. While this has the disadvantage of diluting the sample (20%) it prevents stripping of volatile components and prevents degradation of labile components prior to column introduction.

To test the affect of added organism and the influence of a saturated soil system (as envisioned in an in situ recirculation zone) it was necessary to maintain the site water at the oxygen levels theoretically attainable in the treatment/ oxygenation zone of the well. This treatment zone is represented here by the treatment columns tested in the field. To model this, oxygen was added indirectly via dilution of the site water with oxygen saturated water at a ratio of two parts contaminated water to one part oxygenated clean water. Potable tap water was aerated on site and combined with the inflow of untreated contaminated site water from the barrels. The effluent was monitored for oxygen content over the course of the three treatment phases outlined above. These levels are presented in **Figure 71.**

This level of oxygenation was sufficient to maintain oxygen output levels between 3.5 and 15.3 mg/L in the uninoculated control column, but was considerably lower for the inoculated columns, particularly the one inoculated with $PR1_{31}$. The obvious first conclusion would be that the inoculated columns were better able to utilize available oxidizable carbon and thus experienced a greater oxygen demand. Why this should be the case (especially given the acclimation time, carbon additions and heterotrophic bacterial populations found in these columns) is not at all evident. The highest DO levels recorded for the uninoculated column correspond to the introduction of WHF7-1 water. This also happened to be the most highly contaminated water by far, exhibiting approximately 450 and 1800 ppb of benzene and toluene respectively. This could indicate an inability of the resident population to oxidize these carbon sources immediately. This would account for the capacity of the inoculated columns to respond more rapidly. The absolute levels of oxygen present are also found in Table 5.

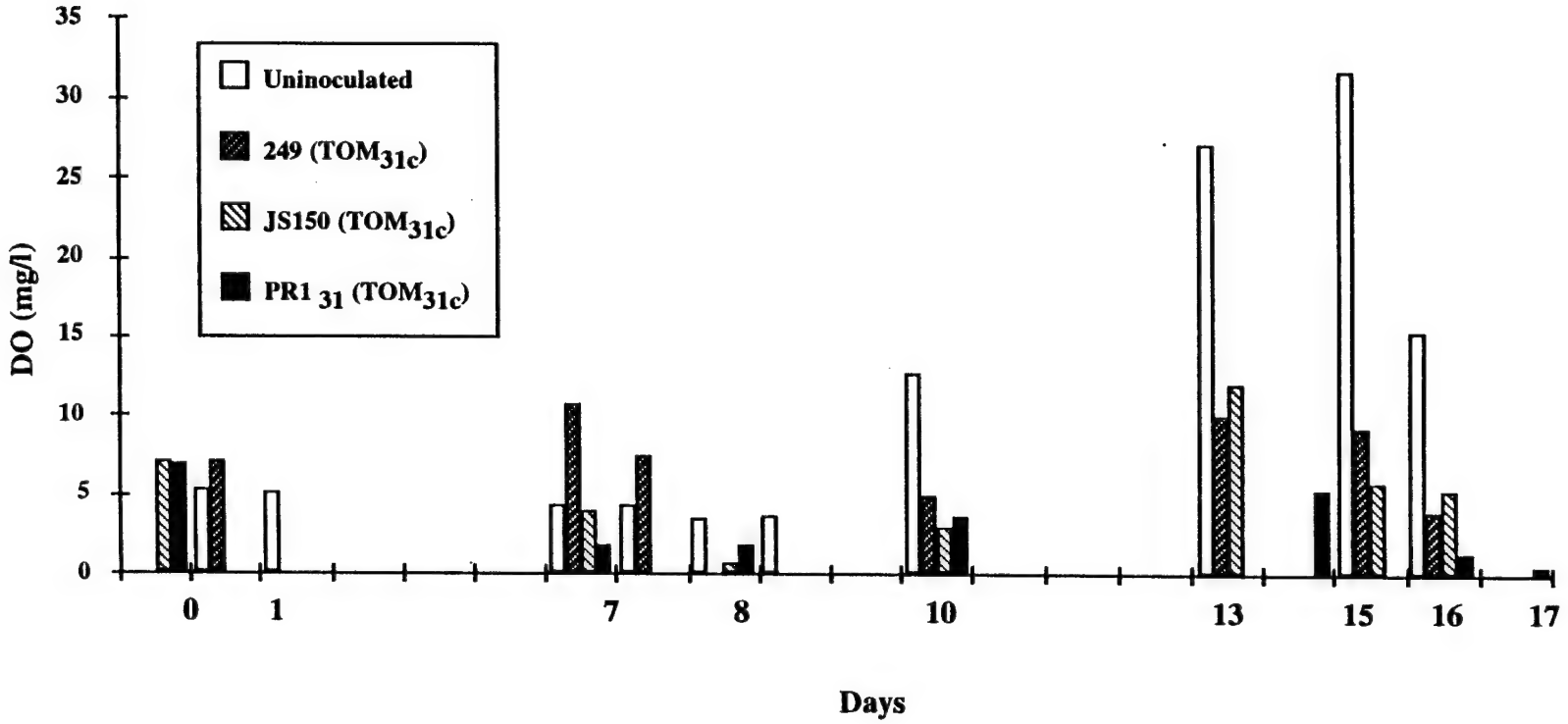


Figure 71. Oxygen Levels of Column Effluents.

Table 5. Soil Columns: Temperature and Oxygen Levels

	Uninoculated			249 (TOM_{31c})			JS150 (TOM_{31c})			PR1 31		
	°C		DO	°C		DO	°C		DO	°C		DO
Day	Hi	Low	(mg/L)	Hi	Low	(mg/L)	Hi	Low	(mg/L)	Hi	Low	(mg/L)
0	27						26		7.1	25		7
0			5.2	26		7.1						
1	28	7	5.1	28	8		27	7		25	7	
2	25	12		24	12		24	12		24	10	
3	24	13		24	13		24	13		24	12	
6	28			27			27			27		
7	28	13	4.3	27	13	10.7	27	13	3.9	26	13	1.8
7			4.3	32	8	7.5						
8	33	8	3.5	29	12		32	8	0.7	33	7	1.8
8			3.6									
9	30	12					28	12		27	11	
10	27	13	12.7	27	13	4.9	27	13	3	27	13	3.6
11	28	18		28	18		28	18		27	18	
12	33			32			32			31		
13	35	14		34	14		34	14		33	13	
13			27.2			10.1			12.1			
14	28	17		27	17		28	17				5.2
15	30	18	31.7	30	18	9.3	30	18	5.8	27	17	
16	22	19	15.3	23	20	4	22	20	5.2	29	18	1.3
17		16			16			16		23	20	0.5
18	29	12		28	12		28	12			16	

3. Treatment Results

a. Water Analysis.

Analysis of the site water yielded several identifiable peaks with the purge and trap/GC analytical method employed. Four of these volatiles were routinely identified through the course of the experiment as *cis or trans*-dichloroethylene (the isomers were not separable under the GC conditions employed), TCE, toluene and benzene. The latter two compounds (particularly

abundant in WHF7-1 well water, would certainly contribute to observed BOD's reported in section 2a (above). GC detectable compounds not recognized as one of the above standards were not identified. In no case did any of these represent a major peak as detected by PID.

b. Soil columns: Uninoculated control, JS 150 (TOM_{31c}), $PR1_{31}$ (TOM_{31c}) and 17616 (TOM_{31c})

The addition of lactate, nitrogen and phosphorus to the columns coincided with the application of WHF1466-13 site water (Figure 70). Nitrogen and phosphorus were the only nutrient amendments with WHF30-3 water; and lactate was the sole nutrient added with WHF7-1 water. Since the input waters varied greatly in contaminant concentrations each phase is presented separately with respect to the given pollutant, and the scale appropriate to that sample, by contaminant:

(1). Trichloroethylene

Levels of these volatile components tended to fall with the withdrawal of water from the respective barrel. This is presumably a function of volatilization to the newly available air headspace within the container as the liquid is removed. Clearly the WHF1466-13 and WHF7-1 materials were more heavily contaminated by TCE (10-50 and 50-100 µg/l respectively) than WHF30-3 (< 10 µg/l). The concentrations of TCE present in the influent and effluents of the respective columns under all treatment conditions are shown in Figure 72. Each concentration measurement represents the means of triplicate determinations. The least activity overall was seen for the uninoculated and the JS150 (TOM_{31c}) inoculated columns. However, both adapted quickly to new levels of contaminants. This was particularly notable in the final treatment phase, receiving only lactate and WHF7-1 water, where an adaptation period of approximately 3 days was required to begin removal of the contaminants to the level seen in the effluent from the column inoculated with $PR1_{31}$ (TOM_{31c}) and 17616 (TOM_{31c}). Due to compression of scale each treatment phase is shown separately:

WHF1466-13 + C, N & P (Figure 73)

Initial TCE levels of 25-50 µg/l were only found in the input water on the first two days. These concentrations had fallen to < 15 µg/l by the seventh day. The same pattern was evidenced for the other contaminants measured in this study (see below). It was interpreted to be due to

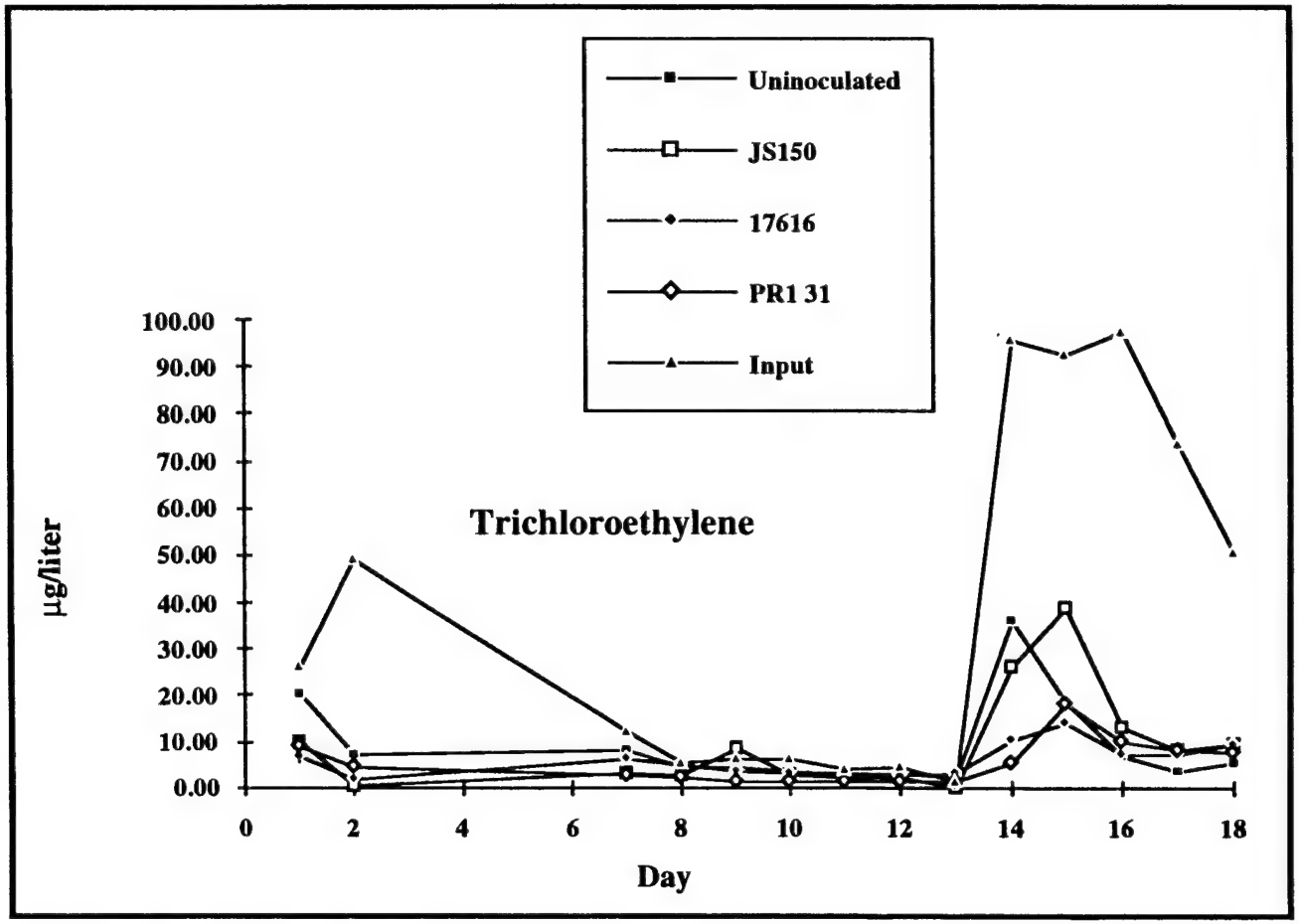


Figure 72. TCE Degradation by Soil Columns in the Field.

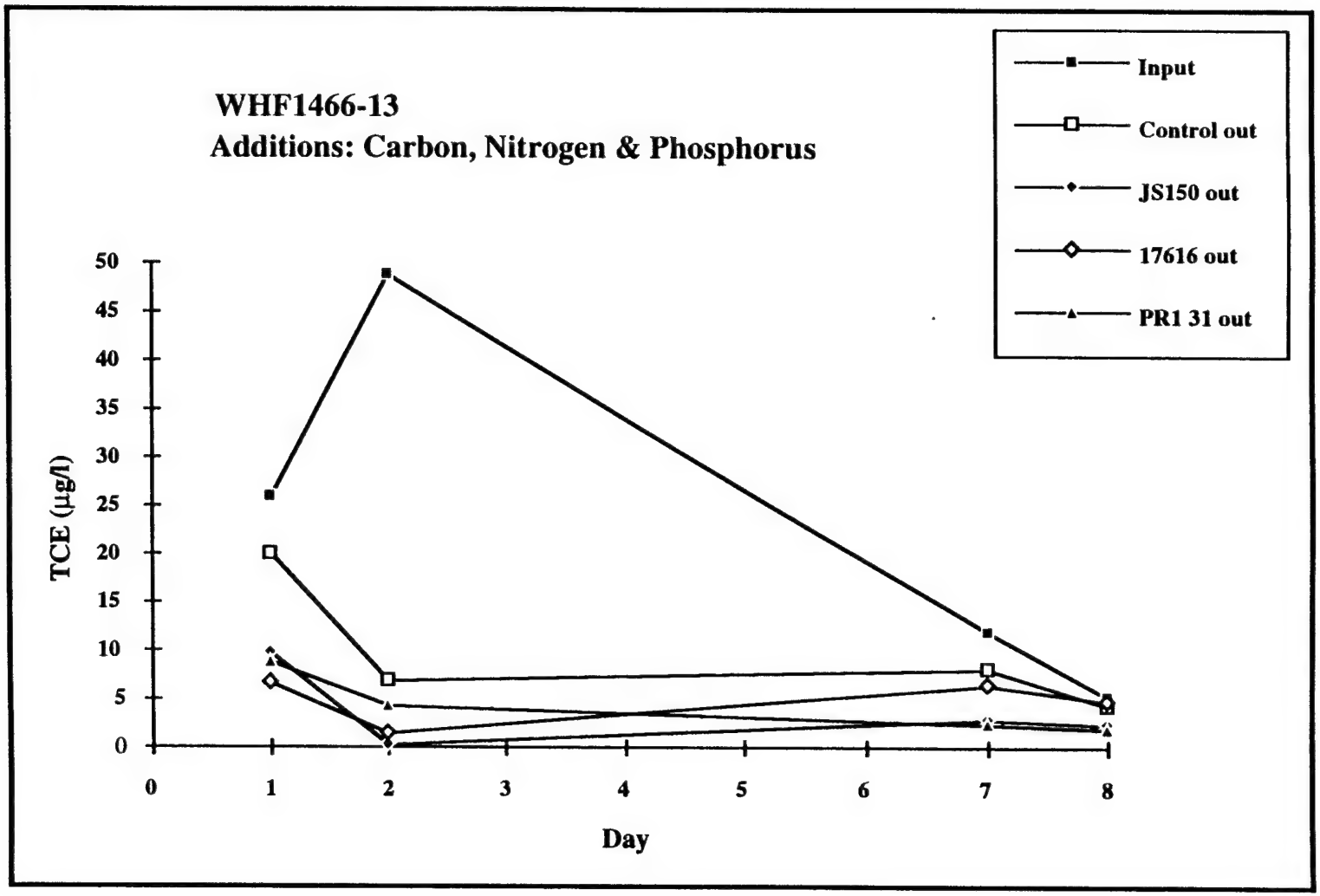


Figure 73. TCE Degradation in Soil Columns Receiving Carbon, Nitrogen and Phosphorus. Sample WHF1466-13.

amendment of the water with lactate and inorganic nutrients (including oxygen), which allowed the intrinsic bioremediation of these chemicals to occur over the relatively long time frame of the experiment. Because of this all subsequent site materials were not amended with oxygen and nutrients until their exposure to the treatment column. All columns demonstrated a relatively efficient removal of TCE at the outset. This included the uninoculated control column. The relatively low levels of TCE present after day two made it difficult to determine if a significant difference existed between the treatment columns. It should be noted that any removal seen would occur during the 8 hour residence in the column, and since the concentration is well below the apparent Km of the enzyme system utilized (i.e. 5-10 μM = 655-1310 μg/l). Therefore at lower concentrations the rate of removal would be expected to slow disproportionately.

WHF30-3 + N & P (Figure 74)

TCE concentrations in the WHF30-3 sample was even lower than that seen in the 1466-13 material (input levels of only 7-2 μg/l). In view of this the scale expansion presented in Figure 11.2 should be accepted as no demonstrable difference in TCE treatment potentials between the four columns.

WHF7-1 + C (Figure 75)

Analysis of WHF7-1 water indicated a similar low level of TCE as that found in WHF30-3. It was therefore decided to TCE to this material. In doing so, an input level of 60 - 100 μg/l TCE was achieved (again falling over the course of the experiment). This steady diminution in TCE levels in the waste barrel could again be due to intrinsic bioremediation (the was the most heavily contaminated sample with two known aromatic inducers of TCE degradation: toluene and benzene), or it may simply be due to volatilization as the air:liquid volume ratio increases as the material is used. This physical change would also in turn provide more oxygen for cooxidative processes.

The $PR1_{31}$ (TOM_{31c}) and 17616 (TOM_{31c}) inoculated columns demonstrated the greatest initial activity, which was maintained for all six days of the experiment, resulting in an approximate 80 - 95% removal of TCE throughout. The uninoculated and JS 150 (TOM_{31c}) inoculated columns were capable of ~70-75% removal the first day, and both had attained essentially the same efficiency of removal as the $PR1_{31}$ (TOM_{31c}) and 17616 (TOM_{31c}) columns by the third day, and maintained this level of activity through the end of the experiment.

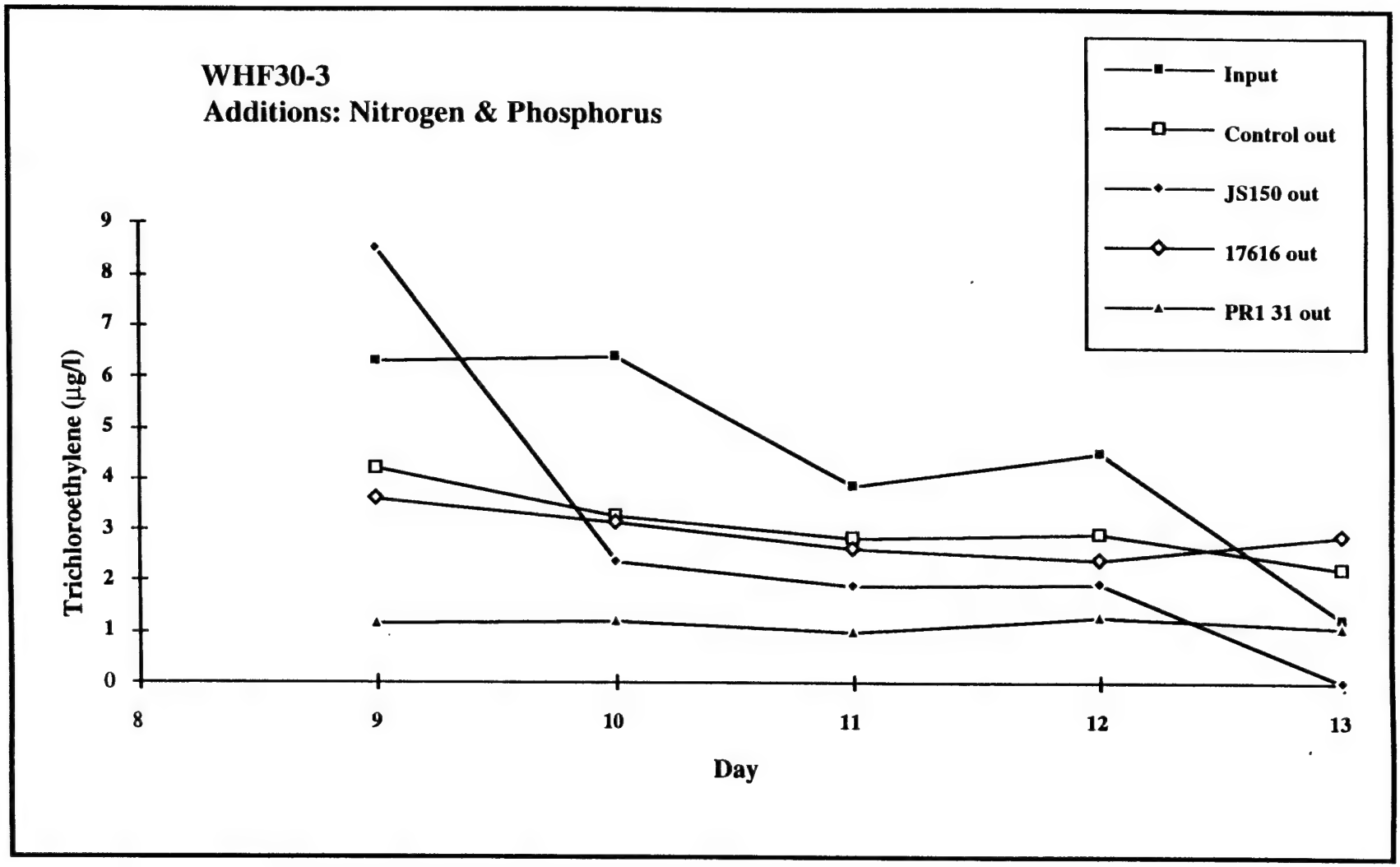


Figure 74. TCE Degradation in Soil Columns Receiving Nitrogen and Phosphorus. Sample WHF30-3

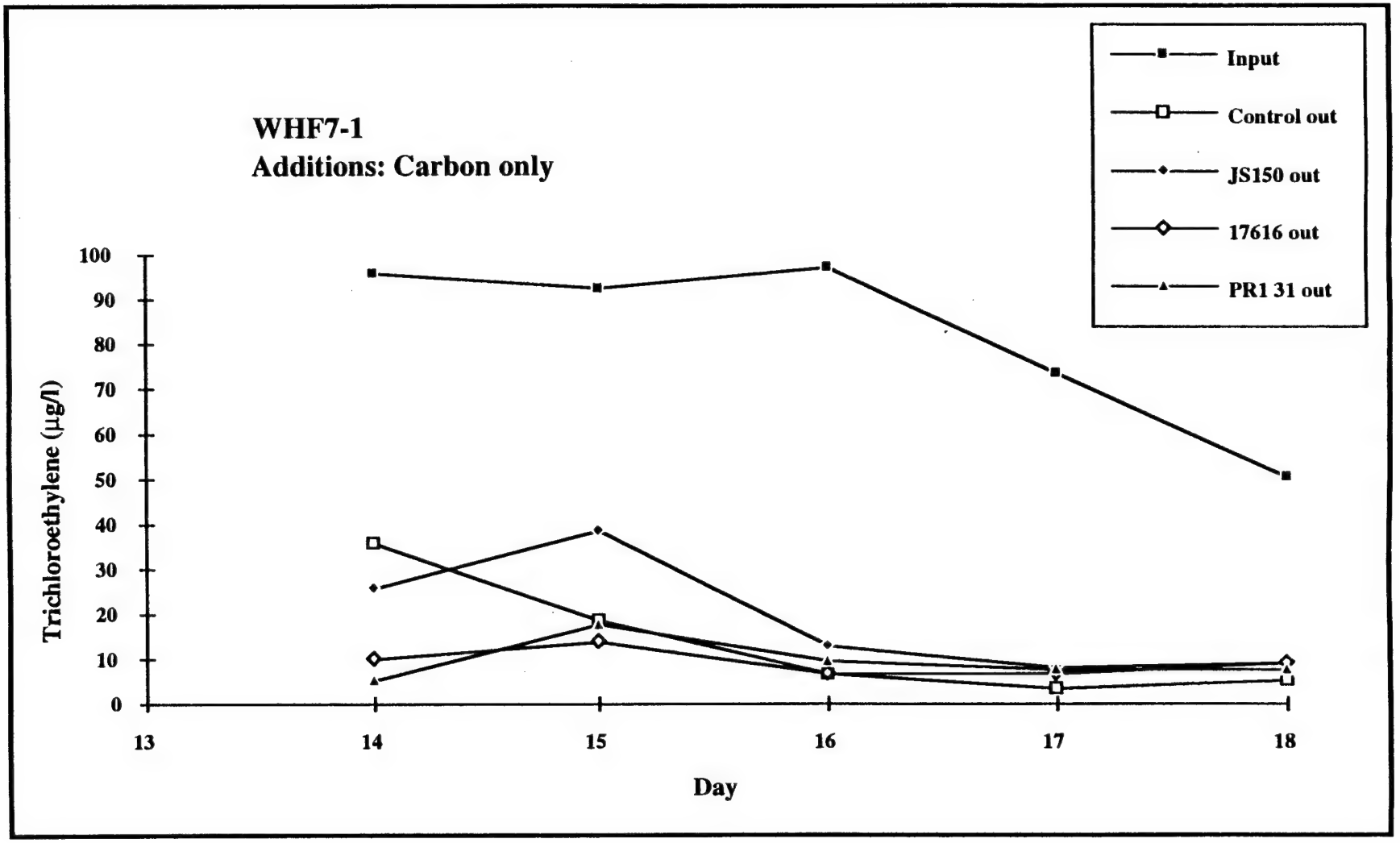


Figure 75. TCE Degradation in Soil Columns Receiving Carbon only. Sample WHF7-1

(2). Dichloroethylene

DCE's were present in the influent materials at much lower concentrations than TCE in all samples. Contaminant removal by the aerated and nutrient amended WHF1466-13 barrel (again presumably due to intrinsic bioremediation) was particularly noticeable for DCE. Such loss was not seen for either of the other two samples (30-3 and 7-1) which were not aerated or amended prior to the treatment column. Influent and effluent concentrations of the DCE's for each of the respective columns under all treatment conditions are shown in Figure 76. Due to compression of scale each treatment phase is shown separately:

WHF1466-13 + C, N & P (Figure 77)

DCE was only detectable in the influent and effluent of one column (17616 (TOM_{31c})) on day one of this treatment period.

WHF30-3 + N & P (Figure 78)

An apparent effect of oxygen and nutrient addition to the 1466-13 material is demonstrated here with the 30-3 sample. The chemical amendments (O_2, NH_4 and PO_4)were not introduced until the material reached the treatment column. This appeared to have a substantial stabilizing effect on the input concentrations of DCE here. Again the levels were very low (2-3 µg/l) in the input, they were stable over the treatment period (5 days) required to pump the entire barrel of 30-3 water through the columns. Only the PR1 31 (TOM_{31c}) column demonstrated any detectable breakthrough during this experiment. This is somewhat interesting given its relatively superior performance with TCE removal at the same time (Figure 74). However, there is really no basis for any conclusion regarding activity since the concentration of output DCE during this test period was found only at the limits of detection.

WHF7-1+ C (Figure 79)

The only addition to the WHF7-1 material (besides lactate and oxygen) was TCE. Therefore this sample was also virtually devoid of DCE with input concentrations were remarkably stable ranging from 0.9 - 1.5 µg/l during the addition of WHF7-1 water. As was the case for the 30-3, N & P amendment test, such low levels allow no conclusions as to its remediation, save to say the treatments failed to reduce the output levels to non-detection.

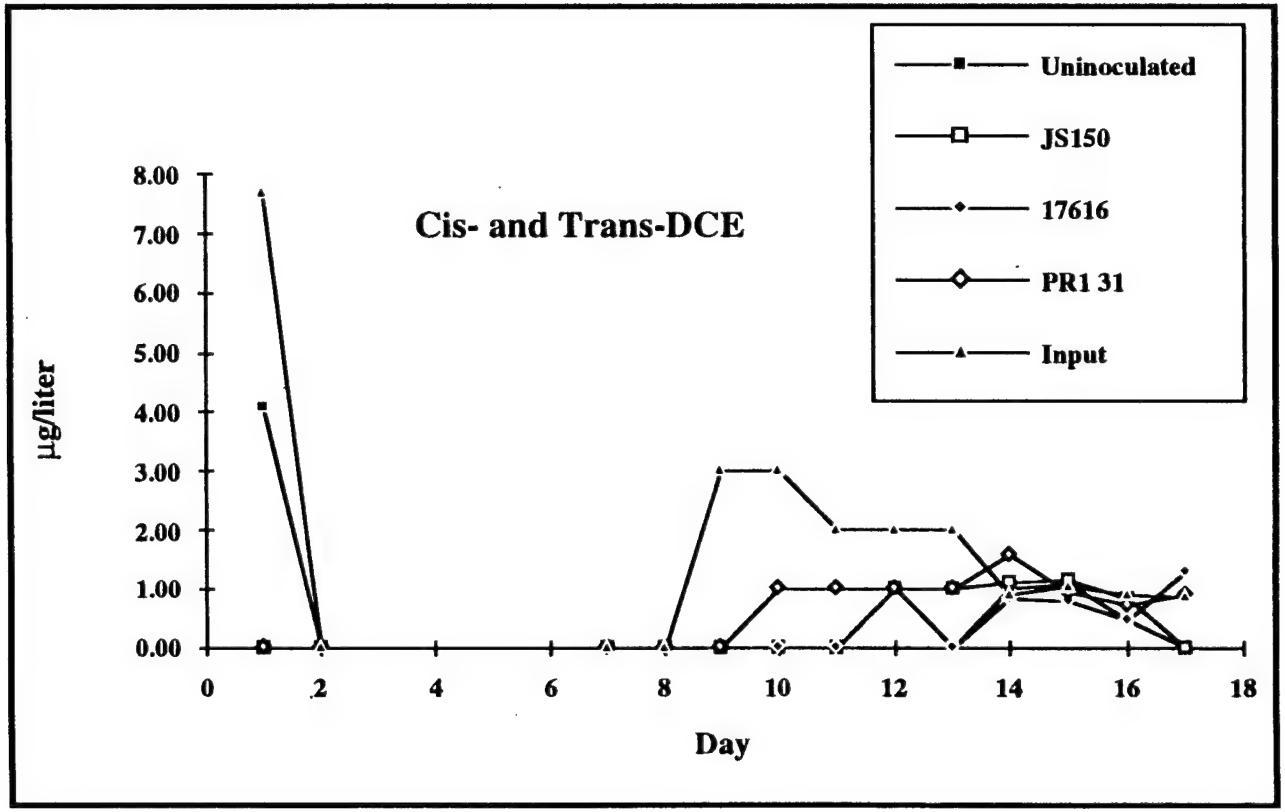


Figure 76. *cis* and *trans*-Dichloroethylene Degradation by Soil Columns in the Field

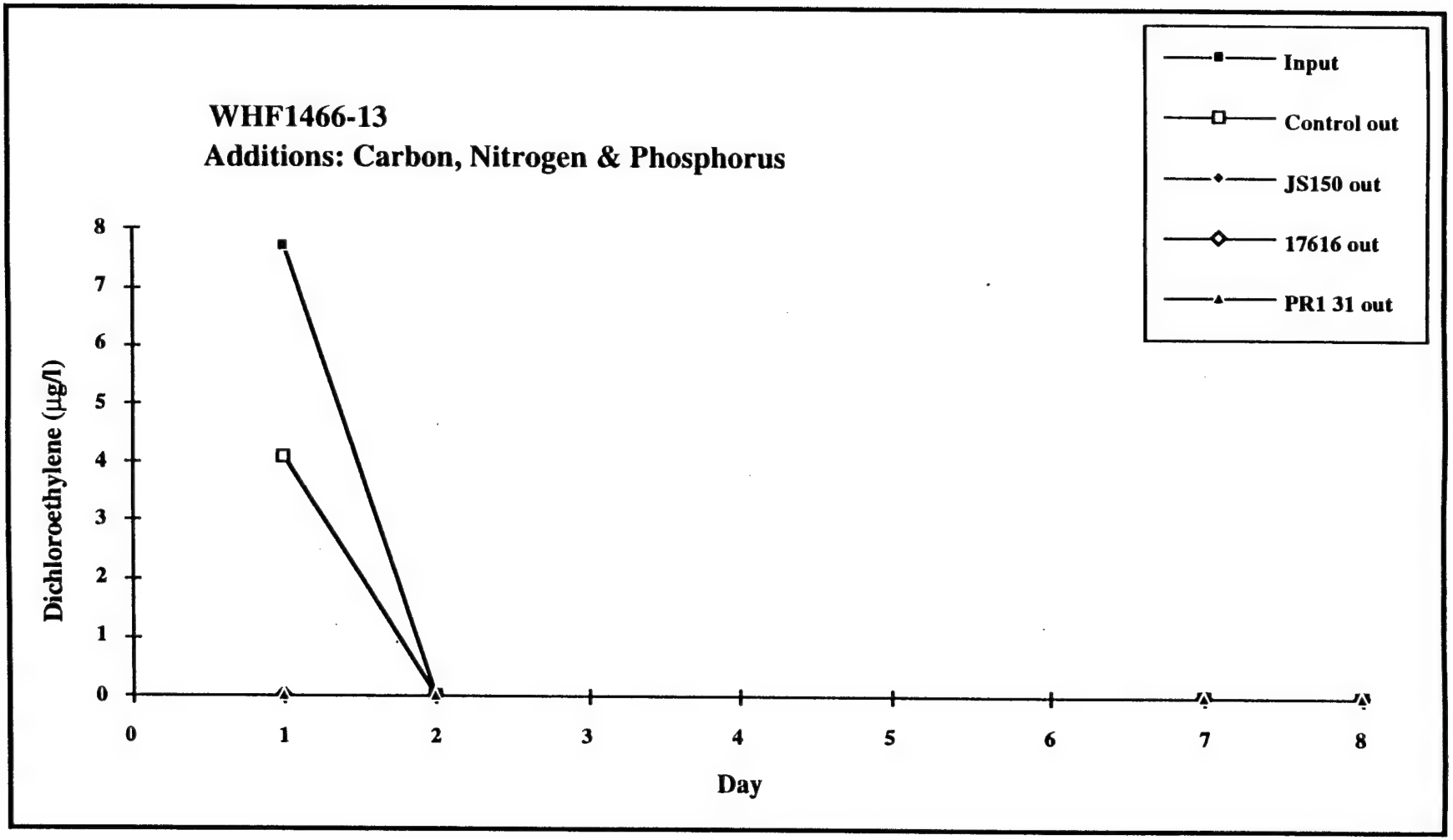


Figure 77. Dichloroethylene Degradation in Soil Columns Receiving Carbon, Nitrogen and Phosphorus. Sample WHF1466-13

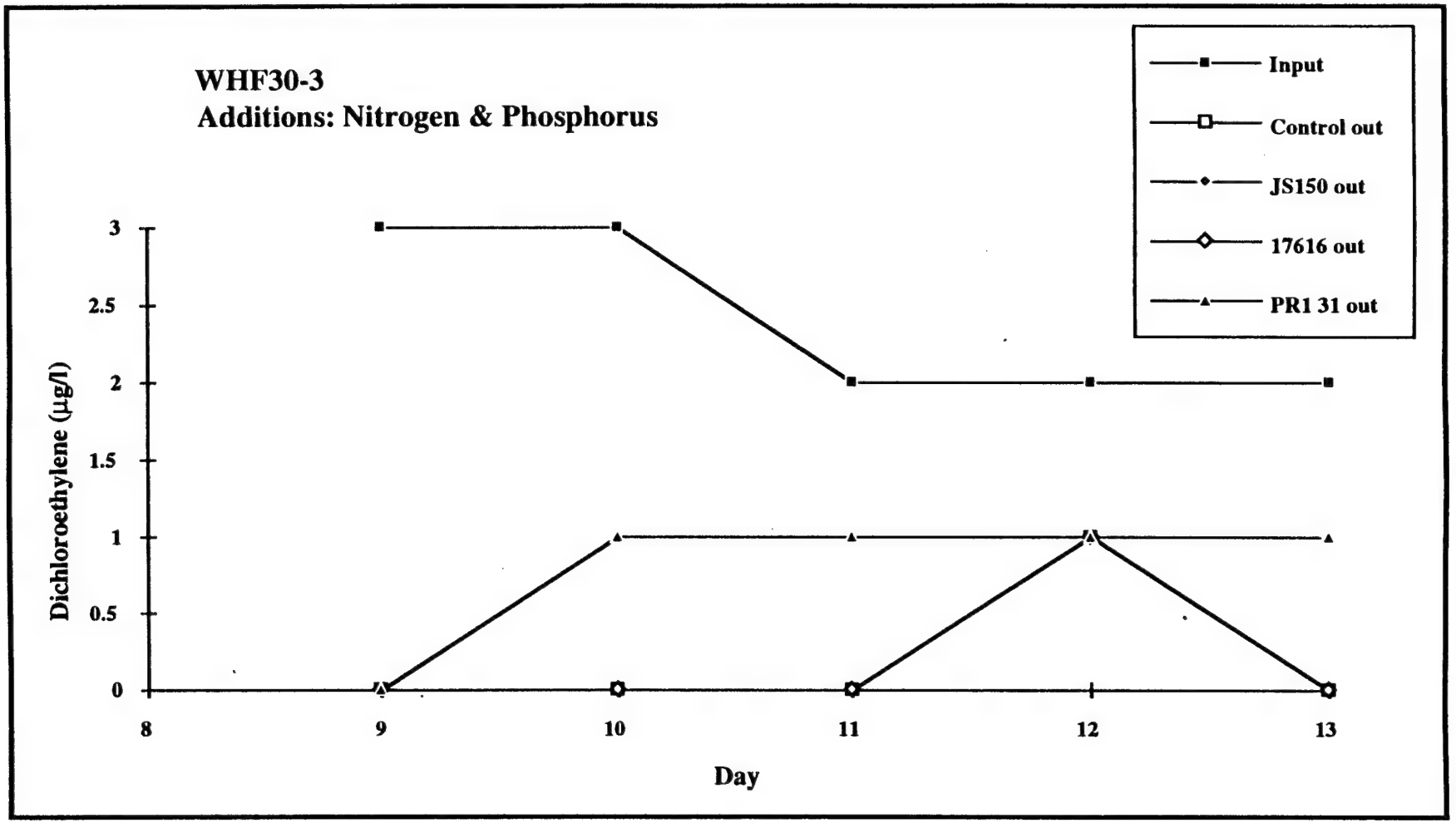


Figure 78. Dichloroethylene Degradation in Soil Columns Receiving Nitrogen and Phosphorus. Sample WHF30-3

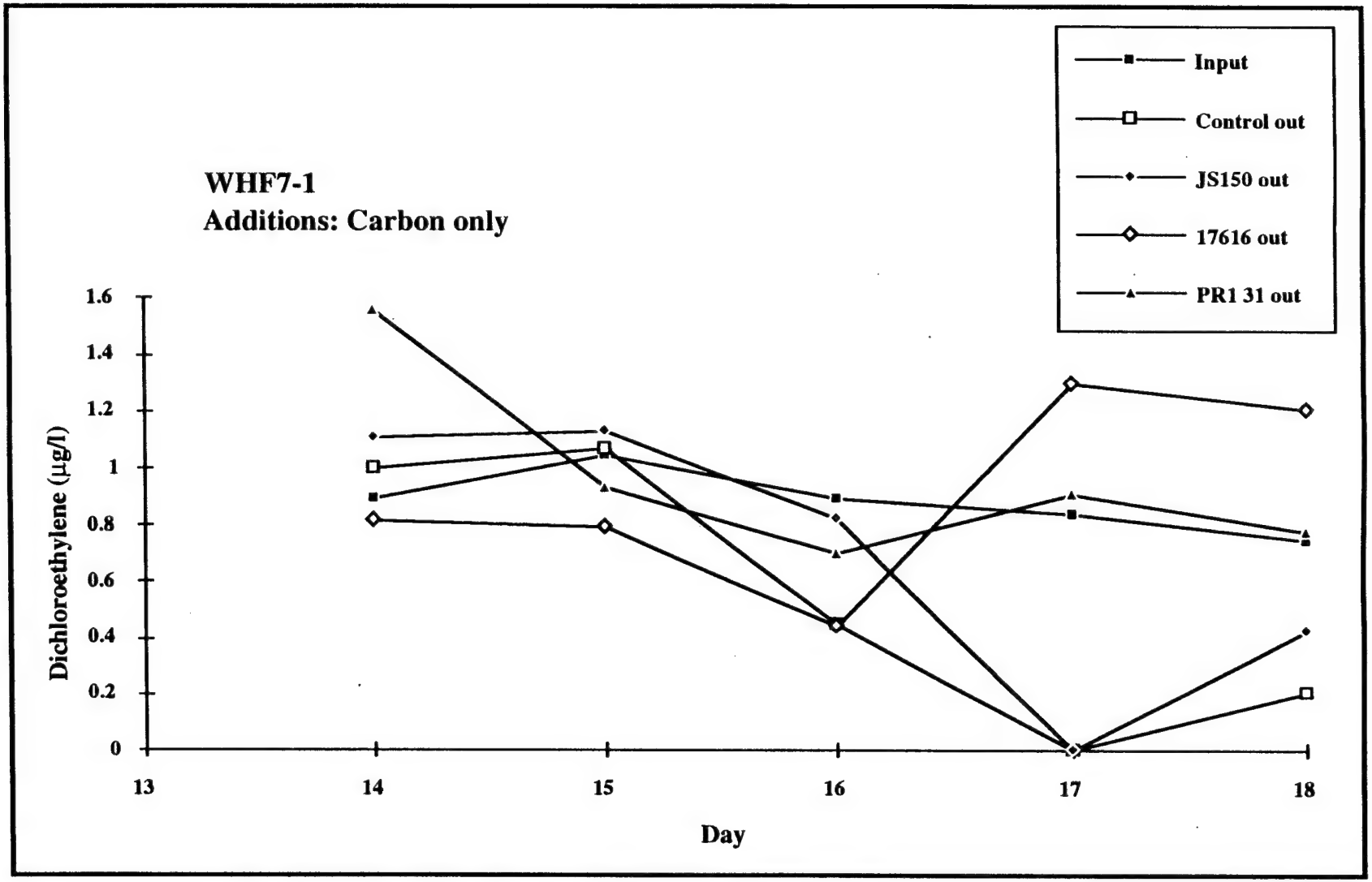


Figure 79. Dichloroethylene Degradation in Soil Columns Receiving Carbon Only. Sample WHF7-1

(3). Toluene

The concentrations of toluene present in the influent and effluents of the respective columns under all treatment conditions are shown in Figure 80. While neither toluene or benzene are targets of the remedial process for which these bacterial strains were developed, they are site co-pollutants with the target compound: TCE, and they are co-substrates for the introduced Tom enzyme activity. Perhaps more importantly, they are known inducers of TCE degradation by bacteria, and as such can serve to induce native populations to degrade TCE irrespective of our addition of constitutive Tom-expressive organisms.

Like TCE toluene was clearly easily removed in the 1466-13 barrel before introduction to the treatment columns. The 30-3 materials contained the least toluene and the 7-1 the most. No obvious effect of amendments (C, N & P; N & P; or C only) were seen on the capacity of the columns to remove toluene as all treatment columns readily removed toluene to non-detectable levels regardless of the chemical additions. Due to compression of scale each treatment phase is shown separately:

WHF1466-13 + C, N & P (Figure 81)

Toluene was only detected in three sample times in this treatment period: days 1 and 2 in the input material, and day one in the uninoculated column output. After this all samples were virtually devoid of detectable toluene. Like the TCE and DCE the oxygen and nutrient amended 1466-13 barrel quickly lost the detectable toluene

WHF30-3 + N & P (Figure 82)

WHF30-3 water exhibited approximately the same initial concentrations of toluene but the input levels were far more stable, gradually falling from ~58 to ~10 µg/l on the sixth and final day of 30-3 water addition. With the addition of only O_2, NH_4 and PO_4 all treatment columns removed essentially all detectable toluene.

WHF7-1+ C (Figure 83)

WHF7-1 was by far the most heavily contaminated material with regard to BTEX. Like WHF30-3 the input levels of toluene from the WHF7-1 barrel remained fairly consistent with a gradual decline from ~1900 to ~1000 µg/l over the five days this material was pumped to the

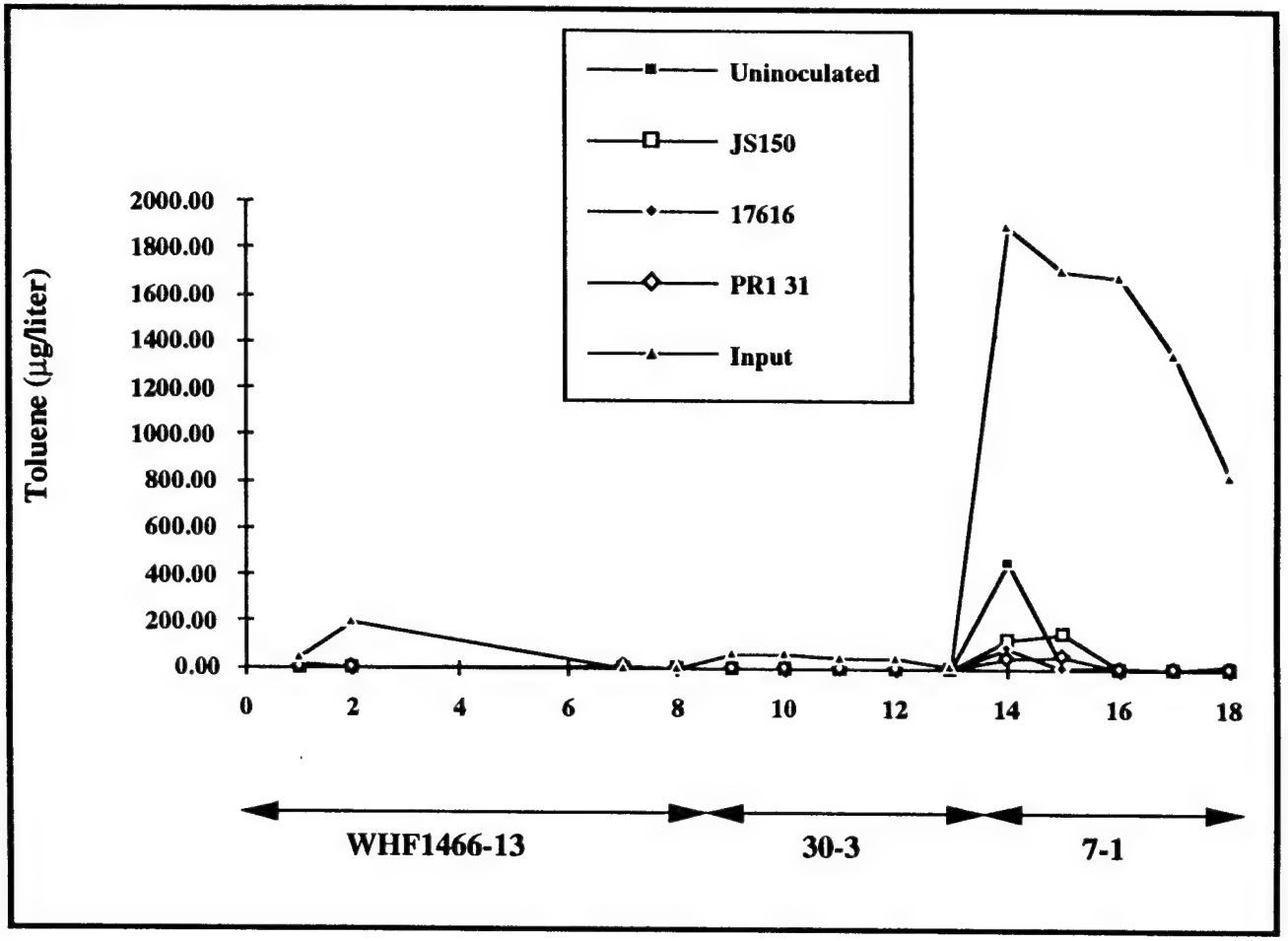


Figure 80. Toluene Degradation by Soil Columns in the Field

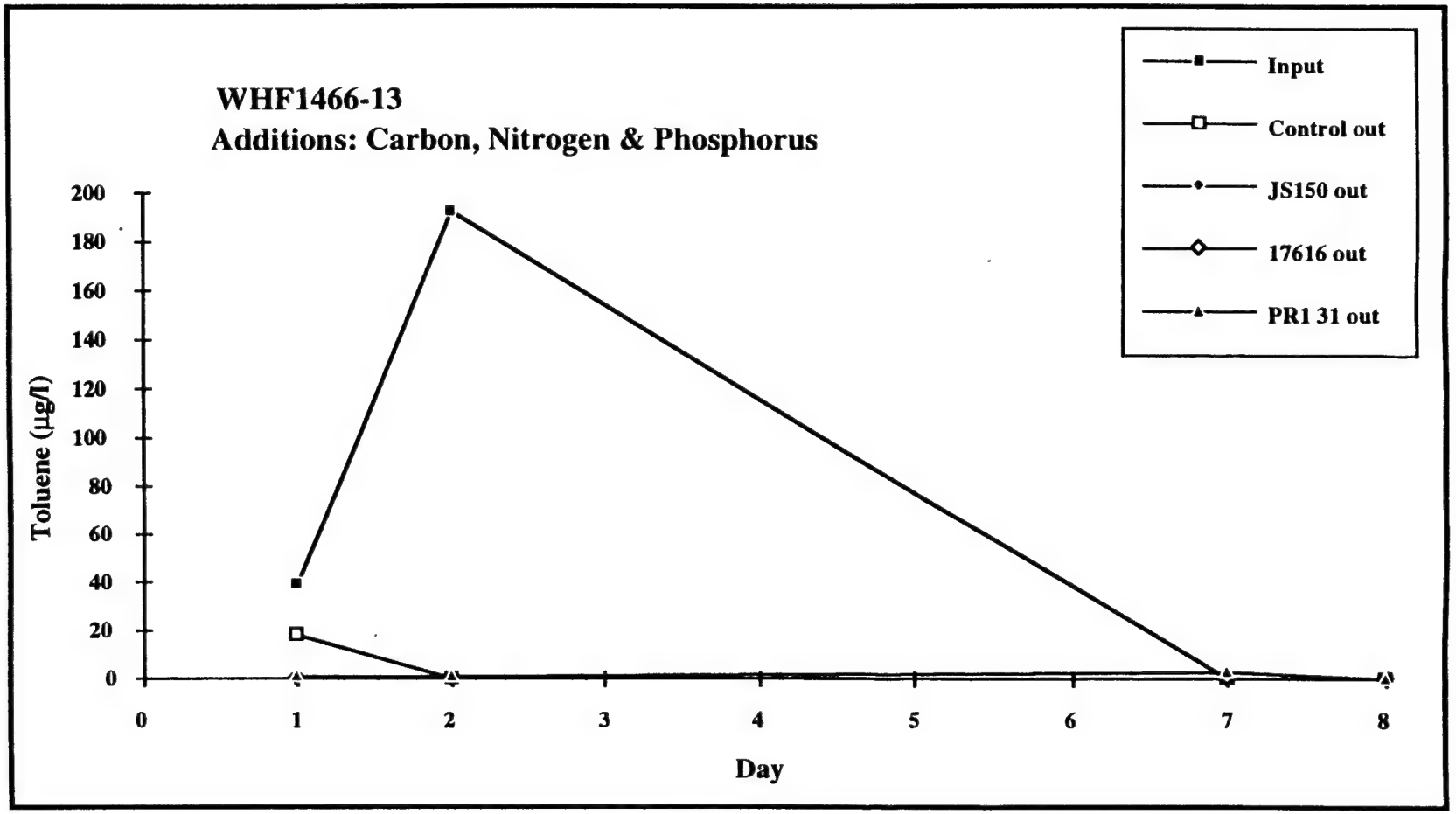


Figure 81. Toluene Degradation in Soil Columns Receiving Carbon, Nitrogen and Phosphorus. Sample WHF1466-13

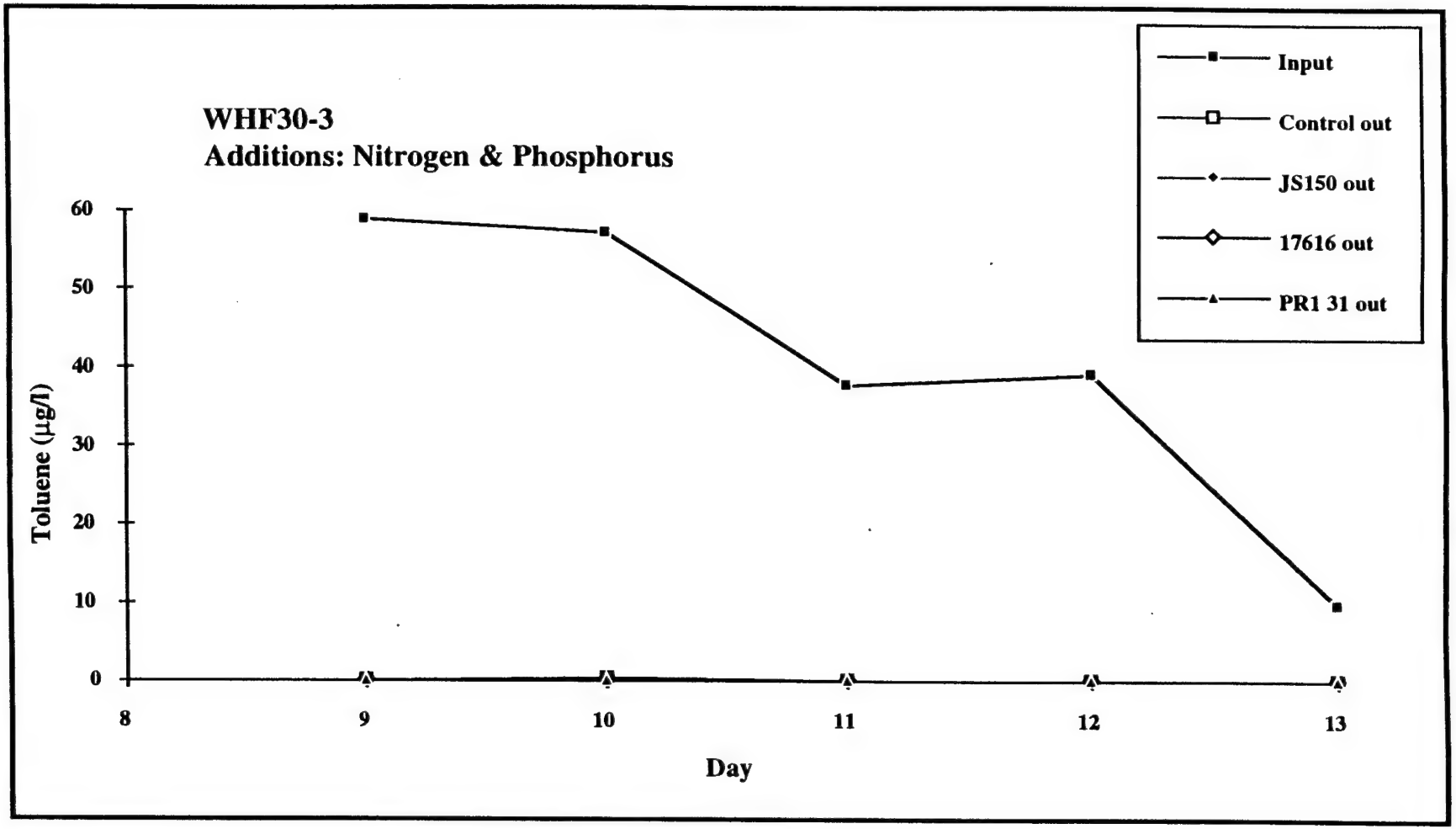


Figure 82. Toluene Degradation in Soil Columns Receiving Nitrogen and Phosphorus. Sample WHF30-3

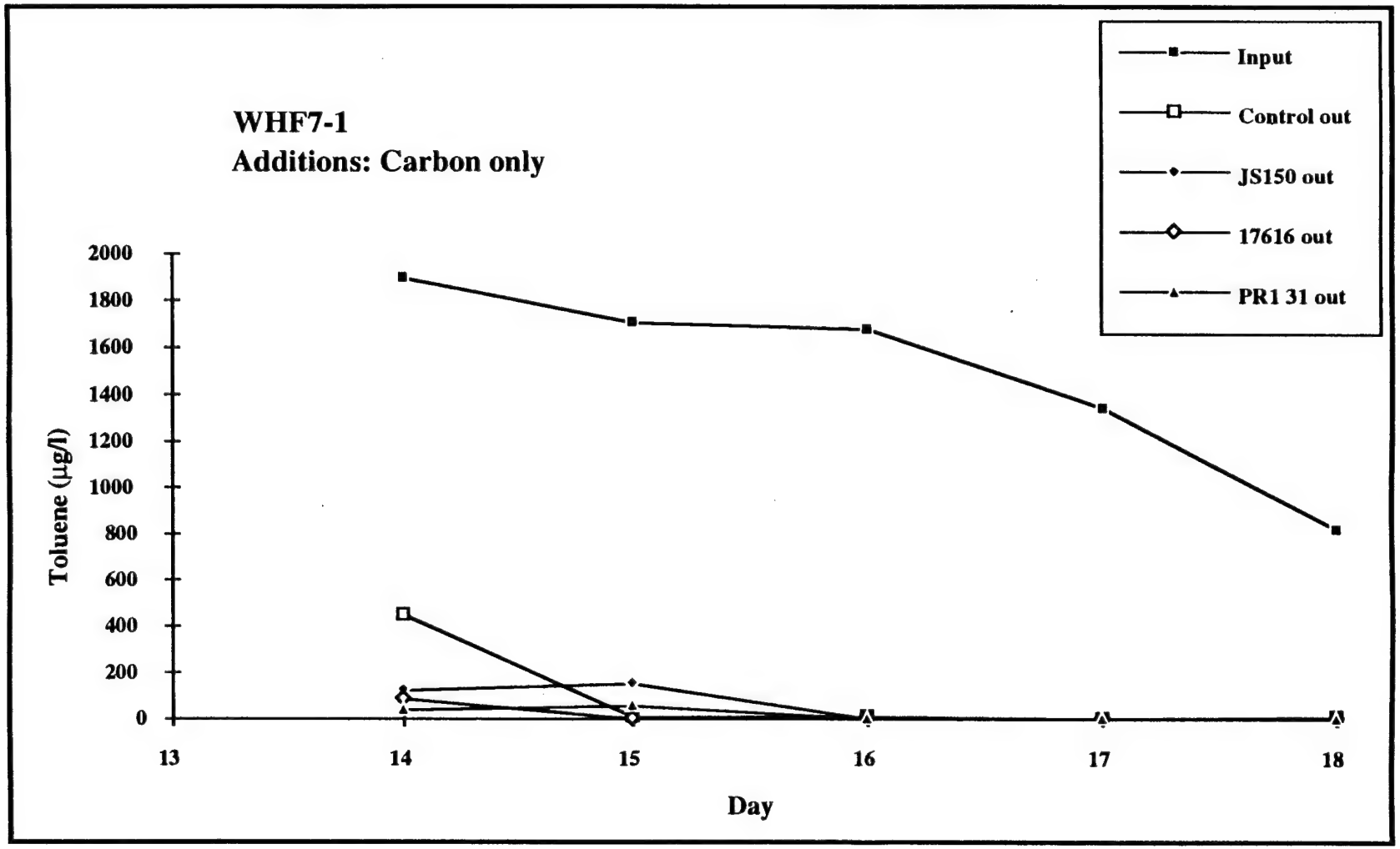


Figure 83. Toluene Degradation in Soil Columns Receiving Carbon Only. Sample WHF7-1

columns. These higher concentrations were also successfully removed during the 8 hour residence time of these columns. In an obvious acclimation the uninoculated and the JS150 (TOM_{31c}) and PR1 31 (TOM_{31c}) columns required two days of exposure to achieve optimal removal to non-detectable levels.

(4) Benzene

Benzene removal by each treatment column precisely mirrored the degradation profiles of toluene. The concentrations of benzene present in the influent and effluents of the respective columns under all treatment conditions are shown in Figure 84. Due to compression of scale each treatment phase is shown separately: WHF1466-13 + C, N & P, Figure 85; WHF30-3 + N & P , Figure 86; and WHF7-1+ C, Figure 87.

The patterns of benzene degradation in each of the treatments was the same as toluene with the exception of concentration. There was proportionally less benzene present in all samples with approximately 15-33, 1-9, and 250-450 µg/l for each of 1466-13, 30-3 and 7-1 samples respectively. The pattern of response was an actual "fingerprint" reproduction of that seen for toluene, and therefore all preceding comments about toluene pertain here as well.

c. Soil columns: BR23 (TOM_{31c})

As mentioned above a coincidental study to generate field application vectors, constitutive for TCE degradation and more selectable under field conditions, was underway in our laboratory. As a result, at the time of the Whiting effort one such strain had been developed and was also tested at the site, in an identical, but smaller column.

Because of these separate feed systems this two column was operated totally independent of the four large treatment columns. A second small column was run at 5% ethanol input and served as an inactive control.

For these reasons these two smaller soil columns are considered separately.

Because these columns were not in place during the first (1466-13) treatment phase BR23 (TOM_{31c}) results will only be presented for the last treatment phase, which received the TCE amended WHF7-1 material.

(1). Trichloroethylene

WHF7-1 + C treatment results are given in Figure 88A. Unlike the large column responses to lactate amendments which resulted in rapid degradation of TCE this column did not respond as

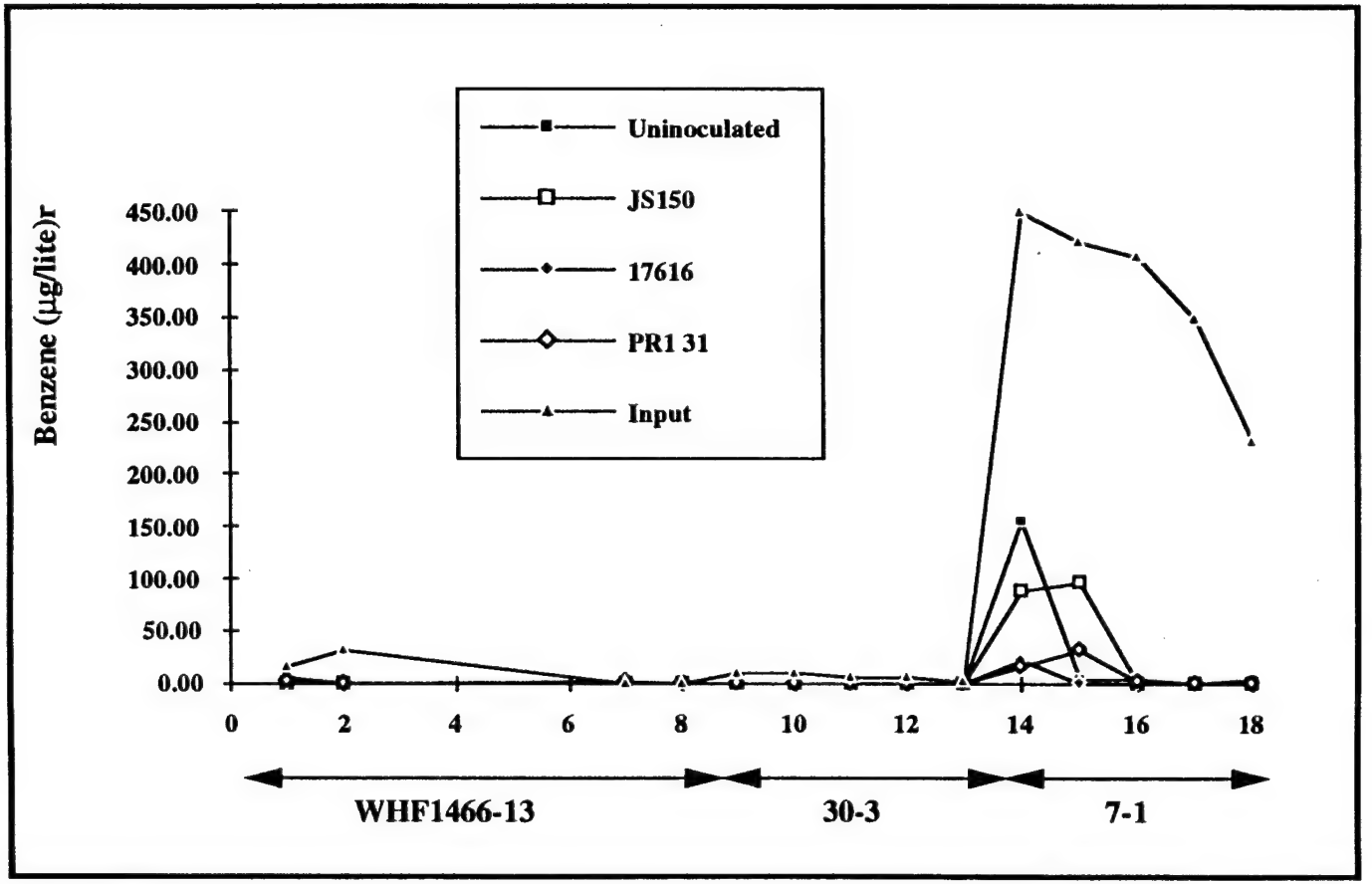


Figure 84. Benzene Degradation by Soil Columns in the Field

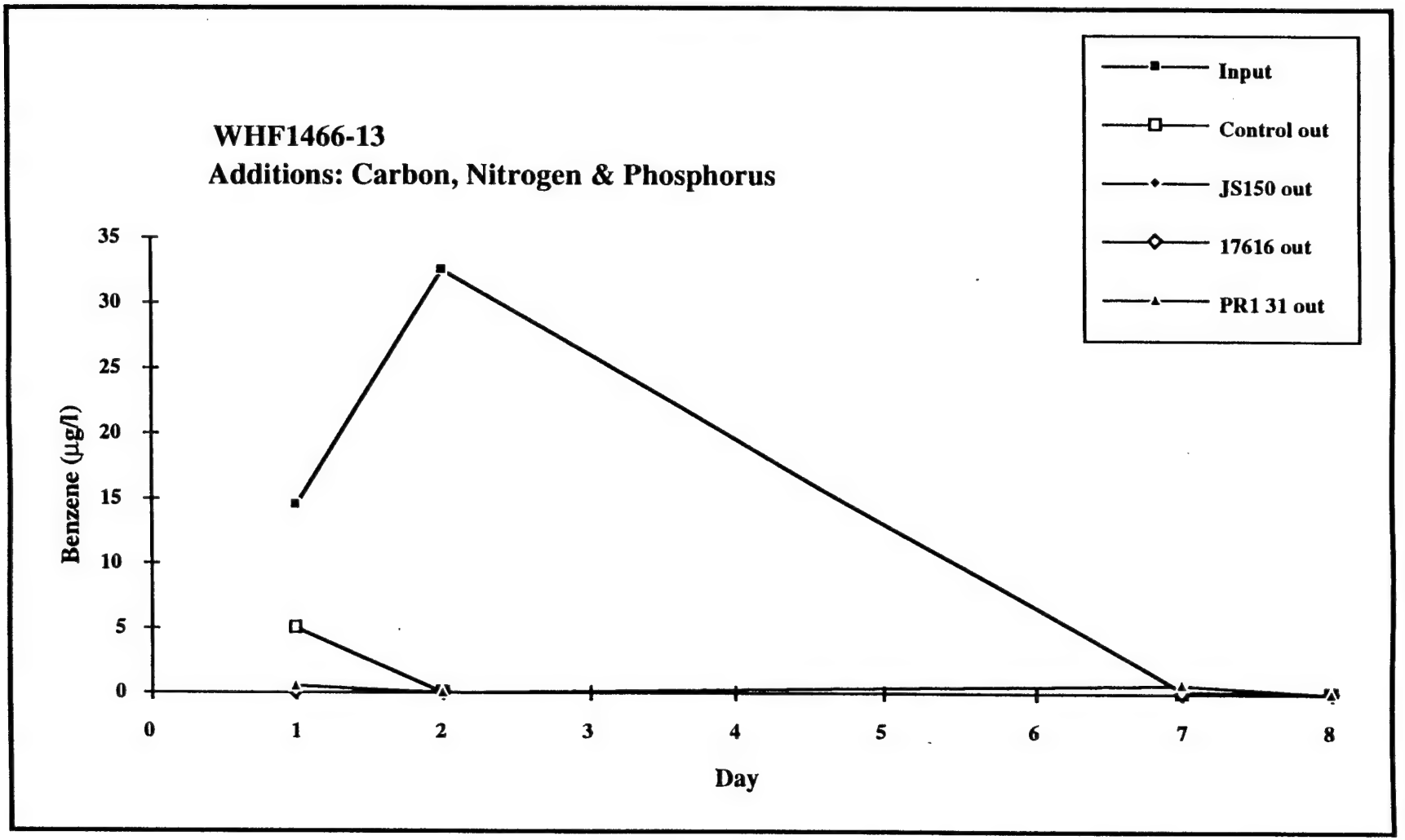


Figure 85. Benzene Degradation in Soil Columns Receiving Carbon, Nitrogen and Phosphorus. Sample WHF1466-13

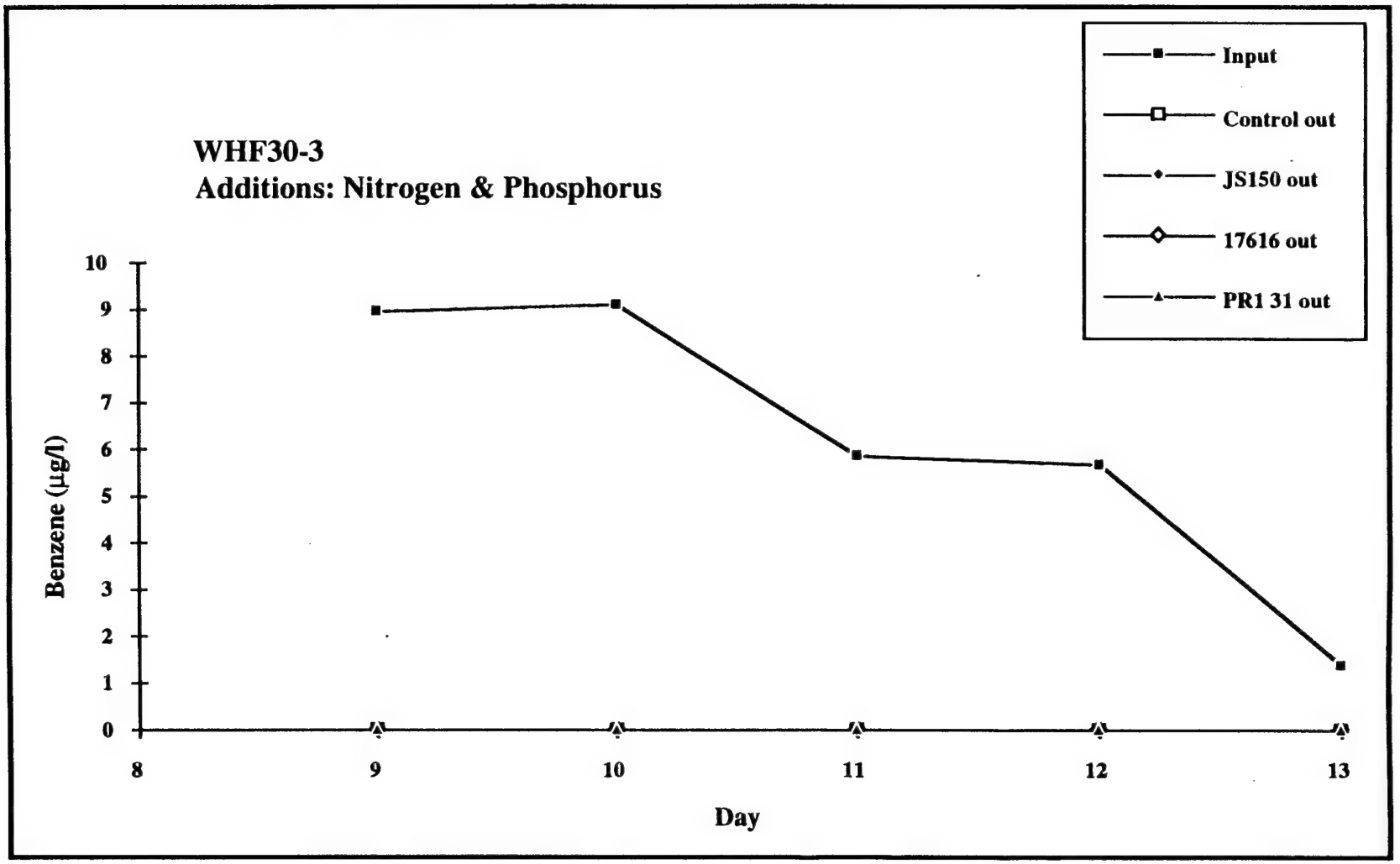


Figure 86. Benzene Degradation in Soil Columns Receiving Nitrogen and Phosphorus. Sample WHF30-3

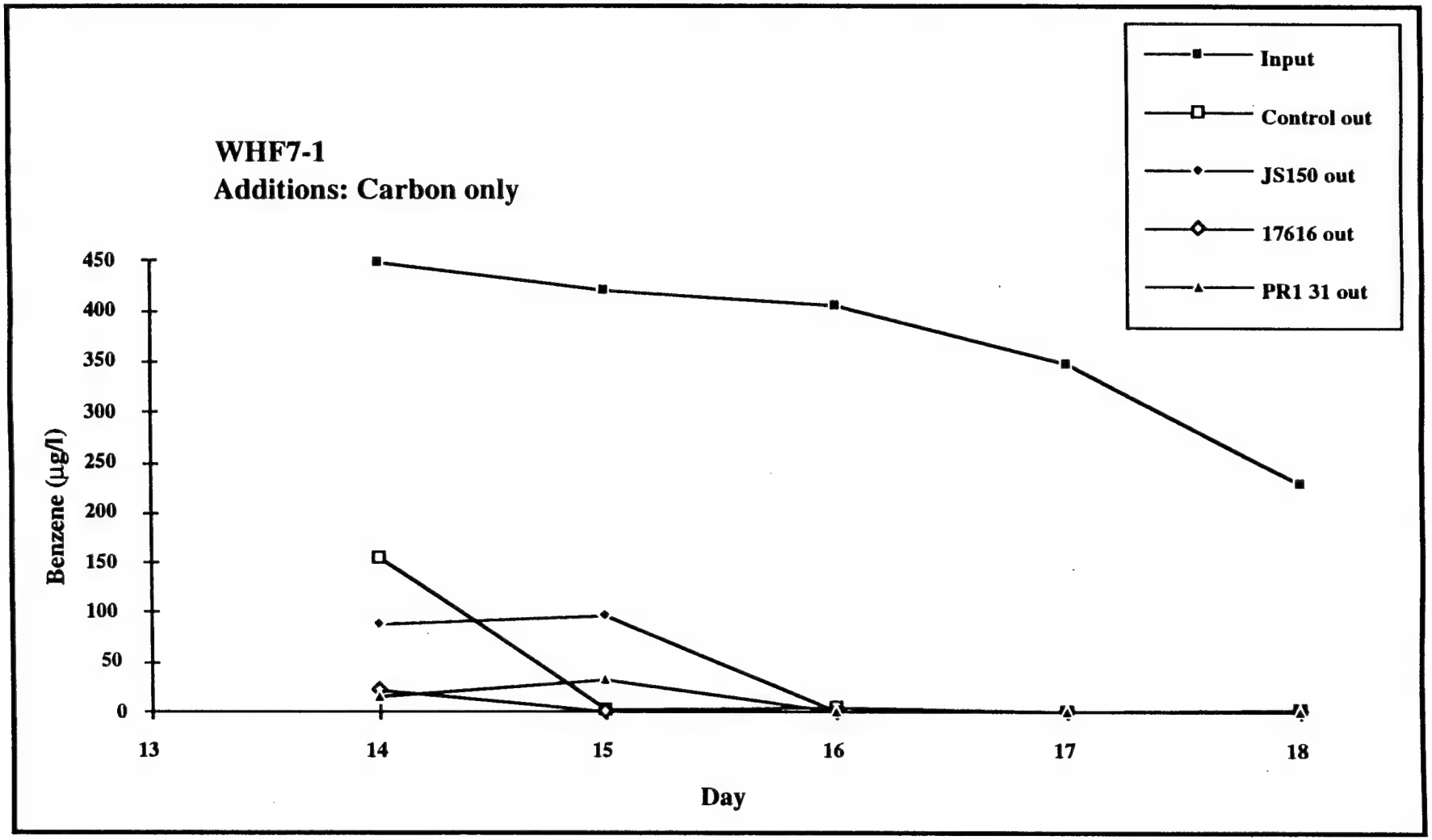


Figure 87. Benzene Degradation in Soil Columns Receiving Carbon Only. Sample WHF7-1

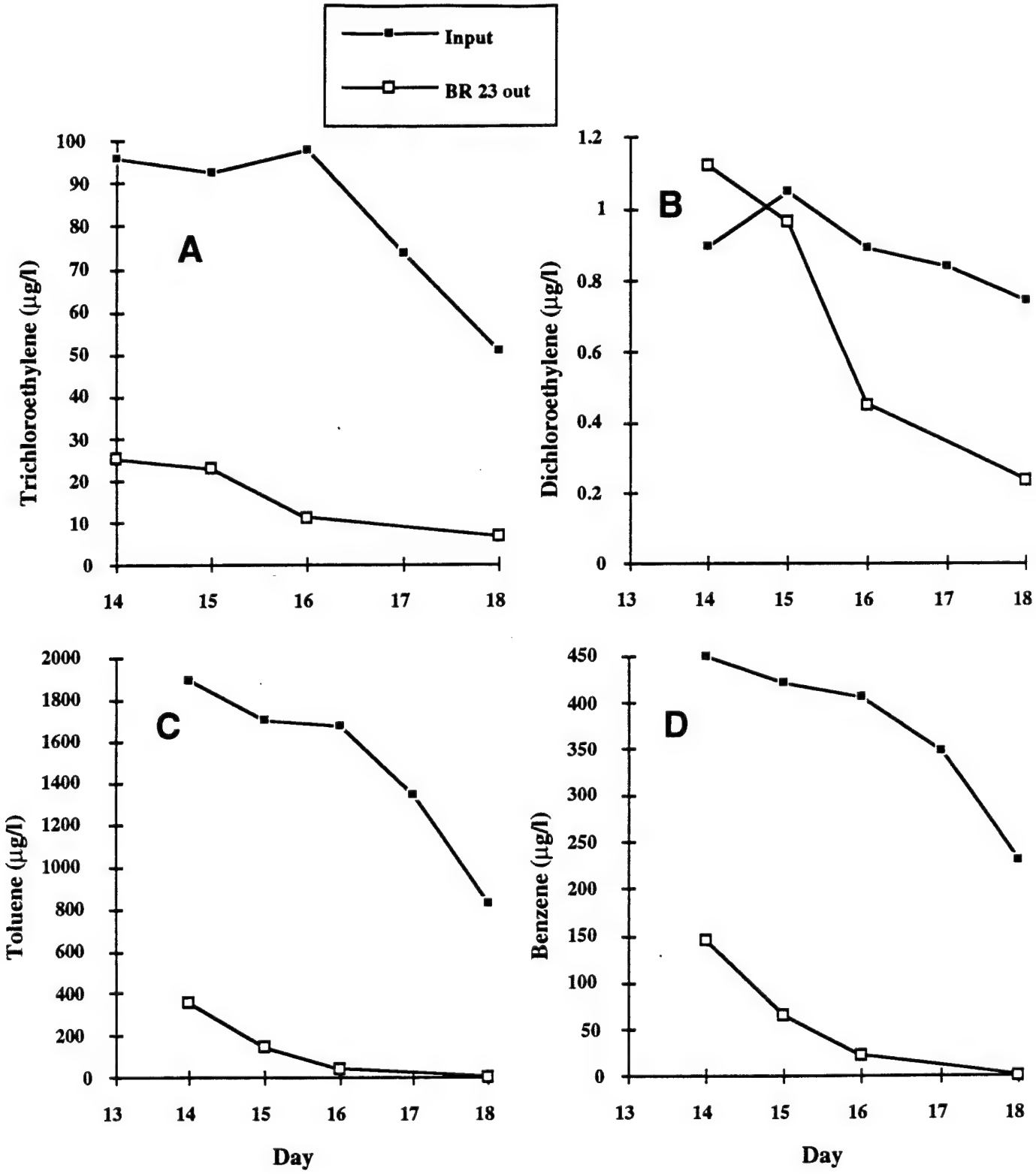


Figure 88. TCE, DCE, Toluene, and Benzene by BR23 (TOM_{31c}). WHF7-1, Carbon Addition Only.

well, with the output levels of TCE tracking fairly well at about 25-30% of the input concentrations (when one allows for the 8 hour residence time between input and output from the column).

(2). Dichloroethylene

The DCE concentrations in this sample were so low as to render the data presented in Figure 88B as unimportant. The behavior was not detectably different from that of the large columns described above.

(3). Toluene

Unlike TCE, toluene in the BR23 (TOM31c) inoculated column was degraded after a 3-4 day acclimation period, as the uninoculated large column described above. This would seem to indicate that despite the potential for toluene induced natural cooxidation of TCE by the native bacteria, this column (physically isolated by an independent feed line, did not exhibit the dual TCE and aromatic "complete" degradation phenomena seen for the large columns (Figure 88C). The column adapted over the final test period resulting in reduction in toluene effluent levels to below detection in the last sample.

(4). Benzene

As documented for the larger columns the degradation of benzene essentially mirrors that seen for toluene (Figure 88D) albeit at lower concentrations, with reduction to nondetectable concentrations by the last sample period.

d. 5% ethanol column

In a separate small column, ethanol was added to the influent water at 5% (V/V). In this column there was no evidence of any significant degradative activity. This column served as an control in which the TCE and aromatic degradation was inhibited for two reasons. One of course is the toxic nature of ethanol at 5%. The other reason, is that even if these bacteria were capable of utilization of this level of ethanol in the soil matrix, there would be a tremendous oxygen depletion associated with its oxidation.

Ethanol was added to the input water source at 5% = 50 g/l ethanol = 50g/l/ 46 g/mol = 1.09 M. Oxygen was present at approximately 8 mg/L or [8 mg/L/32 mg/mmol] = 0.25 mM. According to the stoichiometry of ethanol oxidation each mole of ethanol will require 3 moles of O_2:

$$C_2H_6O + 3O_2 \longrightarrow 3H_2O + 2CO_2$$

The oxidation of 1.1 M ethanol would therefore require 3.3 M O_2 in the water , or ~ 13 times what was actually available. This coupled with the toxic effects of 5% ethanol should effectively preclude bacterial action on these pollutants.

(1). Trichloroethylene

The TCE in the effluent of this test column even more closely followed the input values with 50 - 70% of the input concentration (90-125 µg/l) remaining in the effluent (Figure 89A).

(2). Dichloroethylene

As stated above the DCE concentrations were too low to be an effective measure of activity, but no apparent change in the output concentration was noted (Figure 89B).

(3). Toluene and Benzene

Like the previous examples the behavior of toluene was very similar to that of benzene (Figure 89C & D).

The ethanol treated column failed to degrade TCE, benzene or toluene, thus demonstrating the capacity for this test system to allow recovery of input pollutants over the course of the experiment. It should be noted that due to the continuous flow properties of the column and the 8-12 hour residence times involved that the input and output concentrations would not necessarily be expected to be the same even in a 100% recovery system.

4. Bacterial Community Measurements.

The bacteria present in these soil columns were measured in two ways: total heterotrophs enumerated on a relatively non-selective medium (R2A), and the kanamycin resistant (@ 50 µg/ml) phenol utilizers. The latter population should compose a relative select group to which all cells expressing the *tom* and Tn*5* neomycin resistance genes of TOM_{31c} would belong. It was anticipated that residual background levels of this population would exist in all soils, but we might recognize a substantial increase in those

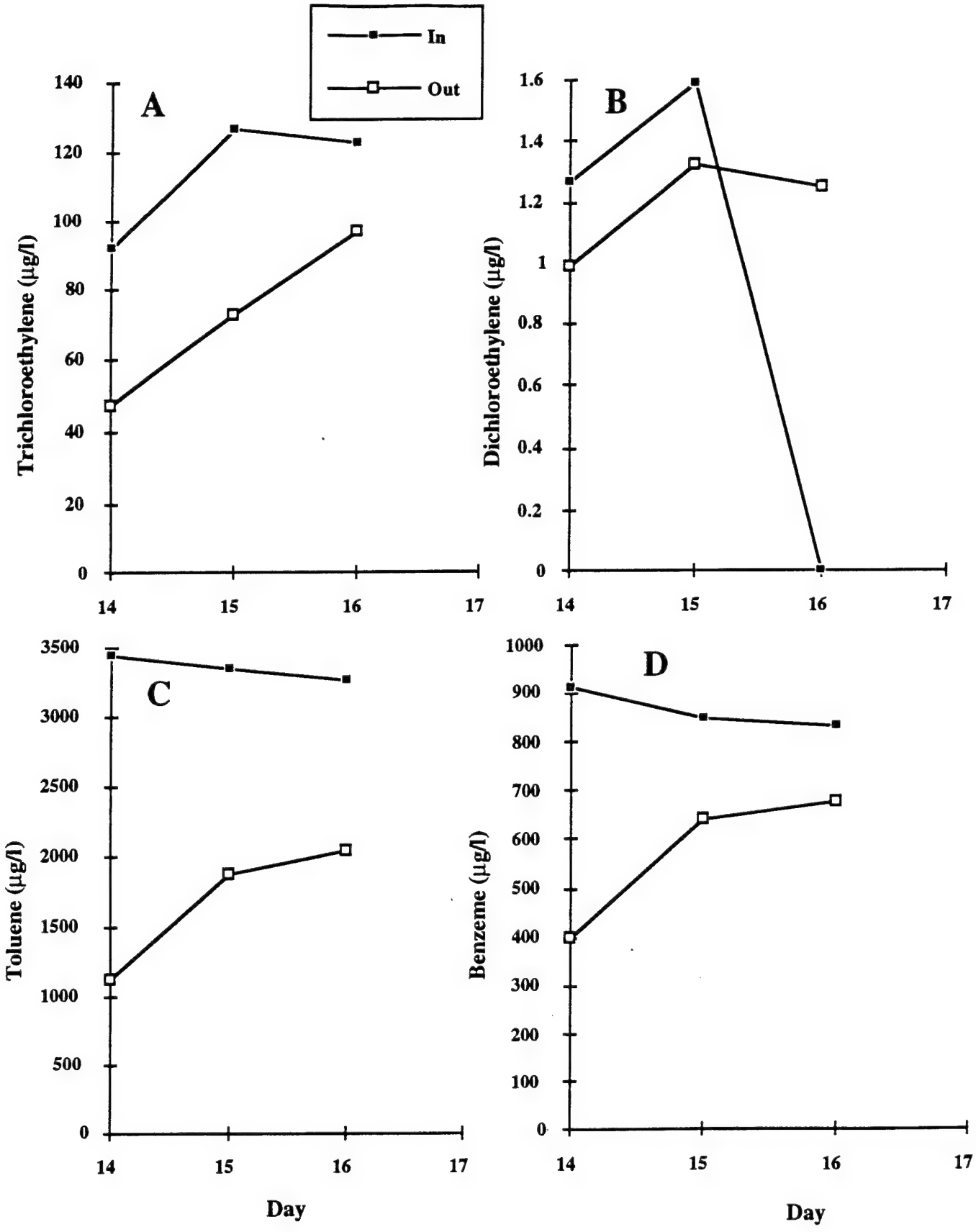


Figure 89. 5% Ethanol Column, WHF7-1, Carbon Addition Only.

where inoculated with TOM_{31c} transconjugants, and this might serve as an indicator of survival and possibly proliferation of the added bacteria. In addition they were measured from two sources of material. One was the column effluent which was monitored throughout the experiment. The other was a soil core taken from each column at the termination of the test. This core was sampled from the center, and 2 cm from either end.

The finding of high populations of kanamycin resistant phenol utilizers in both the effluent and the column packing materials of the "uninoculated" control column have caused us to reevaluate whether this column is actually a "control". Precautions were taken to supply each column with a separate effluent line to ensure that no backgrowth from common effluent lines would contribute to such a cross-contamination. However, but the plugging of the effluent lines, and the possibility of the entire column contents backing up and contaminating the whole common influent line was not anticipated. Such plugging did occur periodically. Whether such mixing occurred in the influent line is not know.

a. Column Effluent Bacterial Populations

(1). Total heterotrophic counts..

The total heterotrophic populations measured from column effluents are given in Figure 90. These populations ranged from 10^5 to 10^7 cfu/ml. This seemed to be relatively stable and not affected by the source of influent water, which was also measured at ~10^6 total heterotrophic cfu/ml (Figure 91).

(2). Kanamycin resistant phenol utilizers.

The only selectable markers present on TOM_{31c} are resistance to kanamycin sulfate (Km) (to 100 μg/ml) and the *tom* genes which encode the utilization of toluene and phenol. Since the groundwater already contained BTEX it was decided to enumerate TOM containing bacteria with and 50 μg/ml Km . There was no reason to presume that preselection of the groundwater communities with BTEX would encourage the growth of phenol degraders of the Tom degradative type. A similar population density of Km resistant phenol degraders was found in <u>all</u> column effluents, including the "uninoculated" control. The lowest concentrations were measured coming from the uninoculated control at 10^2 - 10^3 cfu/ml. The highest effluent bacterial populations were measured from the columns inoculated with $PR1_{31}$ (TOM_{31c}), 17616 (TOM_{31c}), JS150 (TOM_{31c}), and BR-23 (TOM_{31c}) at 10^4-10^6 cfu/ml.

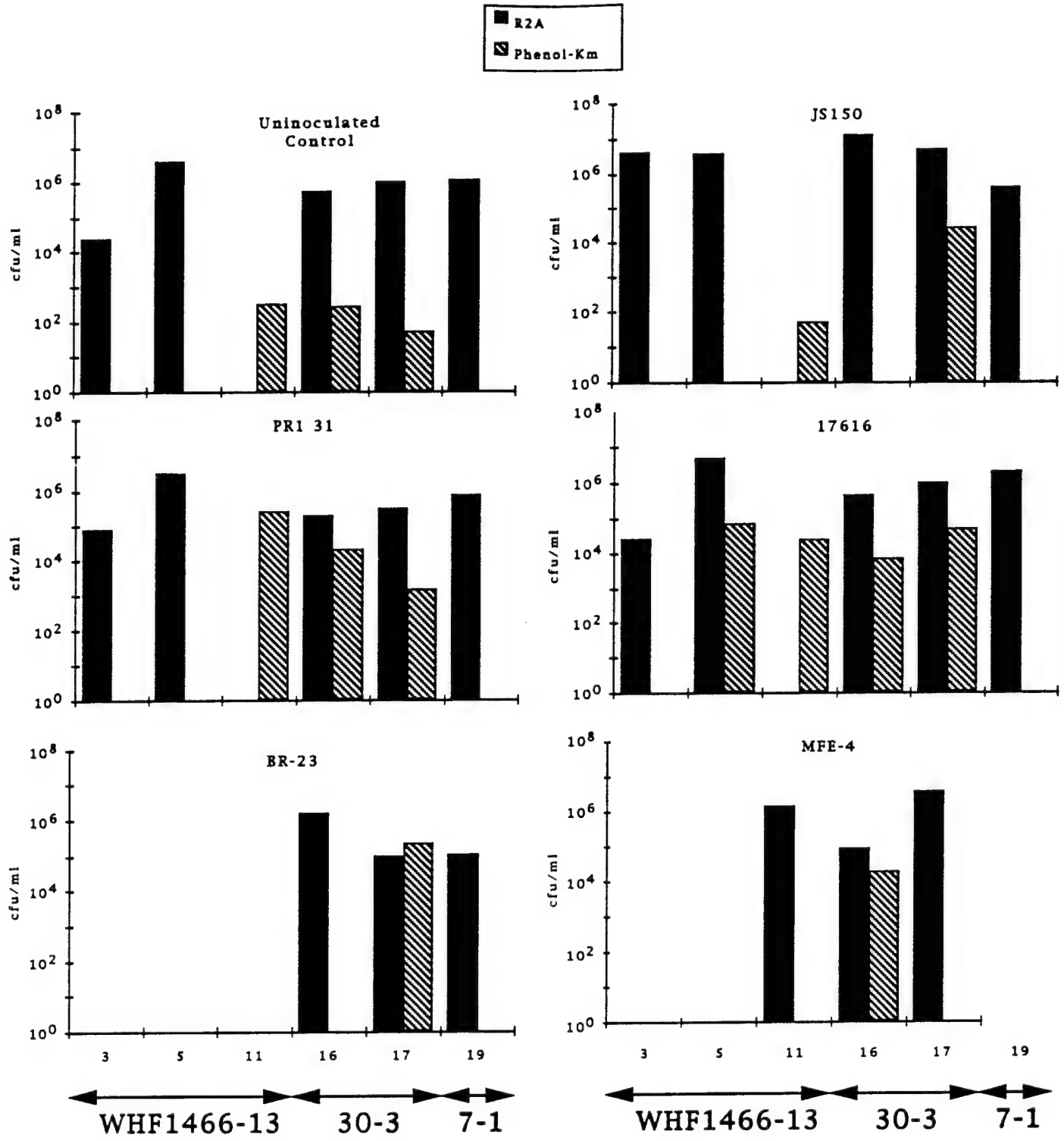


Figure 90. Effluent Microbiological Monitoring of All Columns

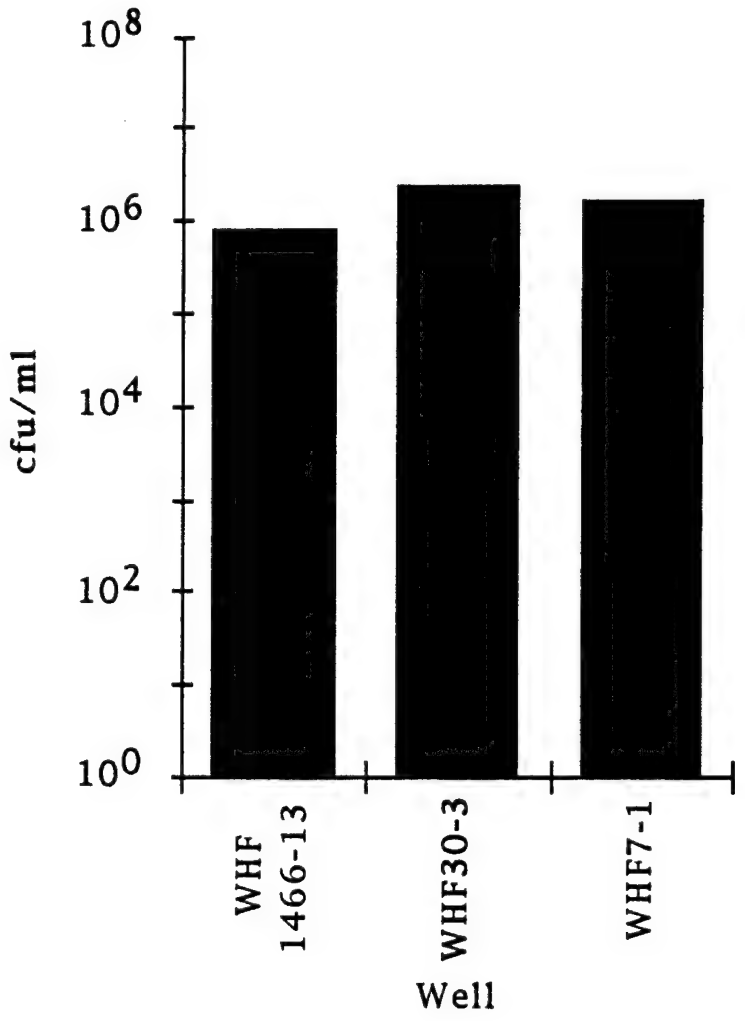


Figure 91. Heterotrophic Bacterial Counts from the Well Waters.

b. Column Soil Core Bacterial Populations

(1). Total heterotrophic counts..

Total heterotrophic populations found in the soil cores (as defined by growth on R2A medium) is presented in Figure 92 for all of the columns at the termination of the experiment. The total heterotrophic populations did not vary significantly among the columns: ranging from 10^6 to 10^7 cfu/ml, which was again similar to the influent water populations (~10^6 R2A cfu/ml).

(2). Kanamycin resistant phenol utilizers.

The only significant trends noted with this population was noted with the $PR1_{31}$ (TOM_{31c}) inoculated soil core. Here the kanamycin resistant phenol degraders made up a substantially greater proportion of the total count. In the lower samples (nearest the source of inoculum introduction) they accounted for nearly all the bacterial counts. They only fell to about 10% of the total counts in the upper 2 cm of the soil core. This was very different for all of the other columns, in which the kanamycin resistant phenol degraders routinely made up only a few percent of the total counts (Figure 92).

Soil used in the construction of the columns was diluted in BSM, and the heterotrophic and kanamycin resistant phenol utilizing populations were enumerated. The heterotrophic population was measured at 4.15×10^6. This is essentially the same heterotroph population density measured for the influent water. The kanamycin resistant phenol utilizing population however was vastly smaller in the native soil: 6.0×10^3. Therefore the source of the 10^4-10^5 phenol/Km populations measured in the control column becomes an important question.

c. DNA:DNA hybridization

Three DNA probes were used to characterize the Km^r phenol-utilizing bacterial populations grown from the soil cores. Colony blots were analyzed via Southern hybridization. These results are summarized in Table 6.

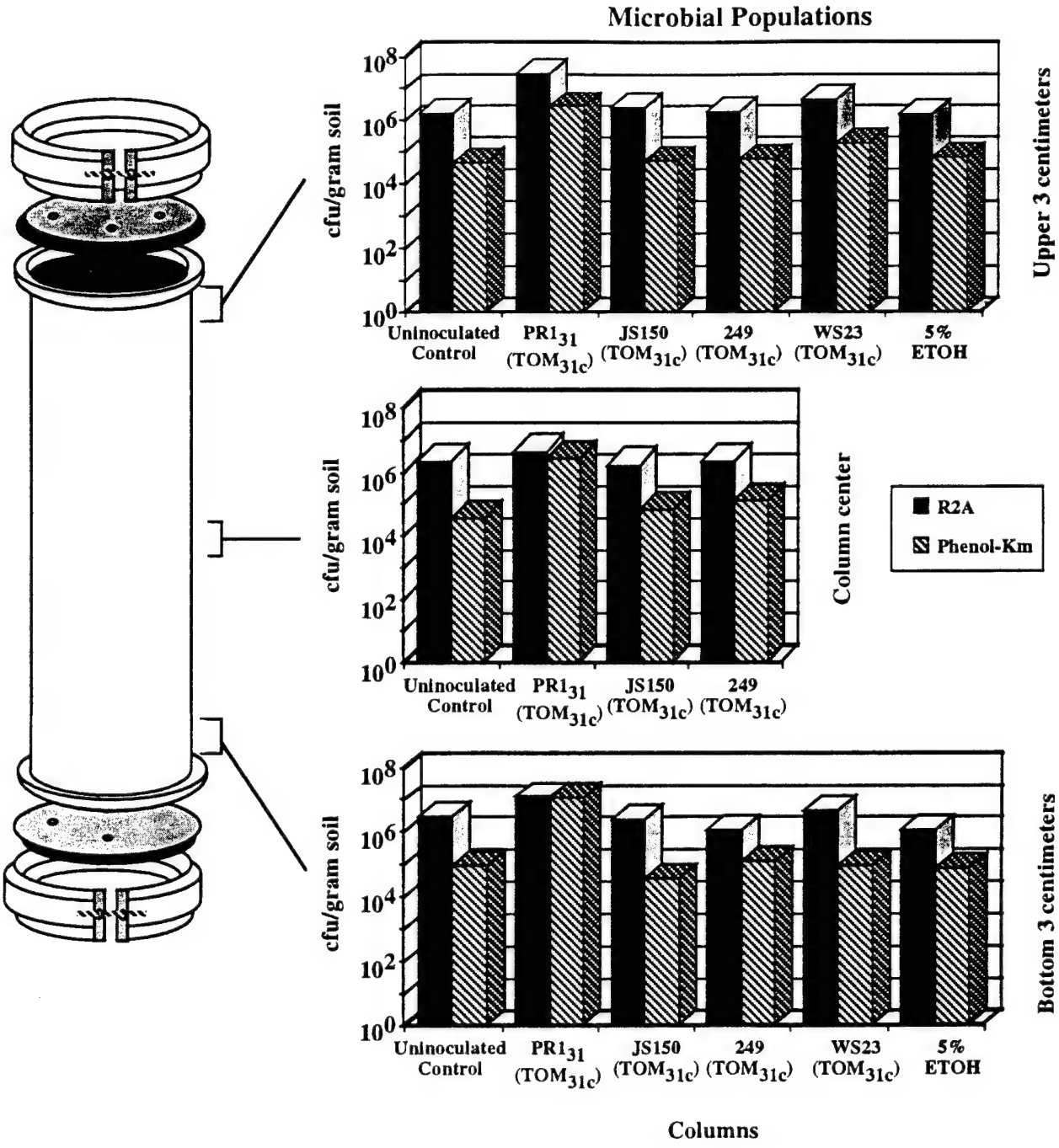


Figure 92. Microbiology of Soil Cores at the Termination of the Whiting Study

Table 6. Colony hybridization response

Column	Sample	cfu	# Colonies Hybridizing to Probe:	% Positive
Control	Hi	62	61	98.4
		41	41	100.0
	Middle	325	284	87.4
		300	321	107.0*
	Low	73	56	76.7
		97	93	95.9
PR131c (TOM31c)	Hi			
	Middle	230	188	81.7
		267	205	76.8
	Low			
JS150 (TOM31c)	Hi	39	35	89.7
		61	60	98.4
	Middle	89	72	80.9
		31	29	93.5
	Low	10	12	120.0*
		53	51	96.2
17616 (TOM31c)	Hi	37	47	127.0*
		76	80	105.3*
	Middle	99	86	86.9
		138	99	71.7
	Low	104	78	75.0
		131	105	80.2

*Percentages greater than 100 are due to small colonies not counted in the original due to the relatively greater ability to detect individual colonies on the autoradiogram compared to the original plate.

Controls: The probe (A *Bam*HI/*Hind*III subclone of *tom*A gene the above fragment containing only the second and third open reading frames of this operon).was found to hybridize to colony blots of all strains carrying TOM_{31c} , but not to the recipient strain BR-23.

A 549 base pair region of IS50L obtained from Tn5 through PCR amplification.

Initial hybridizations with an 11.3 kb*Eco*RI cloned DNA fragment, containing an IS50L sequence, the promoter region of the *tom* operon, the six open reading frames that encode the

*tom*A gene encoding the toluene *ortho*-monooxygenase, and the *tom*B gene encoding the catechol 2,3-dioxygenase) resulted in positive hybridization to all of the phenol/km colonies (at stringencies determined by washes in 1x SSC at 65 °C). The same probe hybridized strongly to positive control colonies containing $PR1_{31c}$ and not at all to plasmidless negative control colonies prepared in the same way and hybridized in the same mixture. This indicated that essentially all these phenol utilizers exhibited homology to some or all of the genes present in this probe.

Because there were so many possible regions of homology the hybridization was repeated with the same colony blots using the *Bam*HI/*Hind*III subclone listed above. This probe also hybridized to most of the colonies (>96%).

Apparently all columns contained either TOM_{31c} or similar sequences (including the colonies grown from the "control column"). Since G4 was isolated from water in Pensacola it is not impossible that the presence of BTEX in the groundwater resulted in the selection of a wild type G4 population at Whiting field. This population would appear identical to the TOM_{31c} containing populations inoculated to the columns with probes I and II. The only real physical difference between the wild type TOM plasmid and TOM_{31c} is the presence of Tn5 and one transposed copy of IS50L in the plasmid.

In order to differentiate between these latter two possibilities, the third probe used is a 2784 bp internal Bgl II fragment of Tn5 (Figure 69) specific to IS50L. While IS50 sequences have been found in environmental samples they are for the most part limited to sources of fecal contamination. It was not anticipated that such sequences should be common in Whiting field materials. Phenol/Kmr colonies from the untreated soil samples were found not to hybridize to this probe, whereas most of the remaining colonies from the test columns did (>90%).

These results would indicate that:

(1) the IS50 sequence is uncommon in Whiting Field materials;

(2) the "control" column did inadvertently receive TOM_{31c}, and

(3) the populations of engineered degrading bacteria used to inoculate these soil columns persisted throughout the test at significantly high levels.

5. Summary of Physical Parameters Over the Course of the Study

The temperature fluctuations of the columns were not controlled but were measured over the course of the experiment. The data are given in Table 5. Because the columns could not be housed in a temperature controlled structure they were subject to the daily temperature fluctuations. The absolute hi and low temperature were monitored daily, and therefore reflect only the extremes. These ranged from a low of 7°C to a high of 35°C. The days were never this warm, but because of a western exposure the columns were subjected to direct sun after 2:00 pm.

D. DISCUSSION

There were no apparent differences in the activity of any of the soil columns with respect to the addition of organic or inorganic supplements. There are several reasons why this may have been the case. One is the apparent oxygen limitation in the $PR1_{31}$ (TOM_{31c}) column throughout the experiment. Another was the fluctuation in contaminant levels found in these site materials. Not only did this represent a variable demand on the cometabolic capacity to degrade TCE, but it also dramatically shifted the amount of carbon source available as toluene and benzene to the introduced bacteria. Therefore, the primary variables manipulated were the addition of bacteria and oxygen.

The most obvious first conclusion is that all of the target pollutants were rapidly degraded in these test soil columns. The most surprising result was the rapid degradation of the same site pollutants in the uninoculated column. There are two completely different reasons for why this may have been the case. Either the indigenous bacteria are fully capable of degrading these compounds because of the co-occurrence of aromatic inducers when provided oxygen; or the control column was not really uninoculated, and in fact actually received a constitutive bacterial inocula.

It is likely that the aromatic co-contaminants in the water would induce the native bacteria to cometabolically degrade TCE. Results from phase II (above), and previous research by ourselves (Shields et al., 1989 and 1991) and others (Nelson, Montgomery and Prichard, 1988) (Hopkins, Semprini and McCarty, 1993) clearly indicate the potential for naturally occurring toluene degrading bacteria to cooxidize TCE. This was in fact demonstrated when the 1466-13 water barrel was then amended with 0.1 mM lactate and BSM. TCE was reduced 40-70% over a one week period, without any bacterial amendments. That TCE is still present in the aquifer may be due to several factors. The plumes may not actually occupy the same space in the aquifer (given the relative density differences) and the water collected was apparently oxygen limited. The influent oxygen concentrations were measured at <2.5 mg/L in the collection barrels. Since no precautions were taken against oxygenation, this level would easily be expected as a result of collection-aeration. Both BTEX components and bacteria capable of their utilization are clearly present in all of the site materials. This would also account for the slightly longer acclimation period seen in the uninoculated and the JS150 (TOM_{31c}) inoculated columns after switching to WHF7-1 water, as the population of toluene degraders appeared to be lower in these prior to the high toluene and benzene exposure (as evidenced by their respective acclimation to higher rates of removal).

Another possibility, is that due to a common influent line, the four large columns all received a mixed inoculum, perhaps due to transient plugging and backflow problems. This is supported by population measurements of the soil cores. The $PR1_{31}$ column (which was immediately adjacent to the "uninoculated" column) had a much higher density of km^r phenol-degraders at the bottom of the column

where the bacteria were introduced. Pre-existing Km^r phenol utilizing populations in the soil used to construct the columns (5×10^3 cfu/g) were found at 5-10 % of the levels seen in the uninoculated control ($0.5 - 1 \times 10^5$). Even at these higher levels however (assuming that these are truly representative of the viable cells present) it is difficult to envision this concentration of phenol degraders being solely responsible for the extent of TCE degradation observed in the uninoculated column over just the eight hour retention time of the soil columns. The fact that almost all of these kmr phenol utilizers hybridize to the *tom* gene probes makes it very likely that such cross contamination did occur.

The maintenance of high levels of km^r phenol-degraders, retaining the plasmid encoded *tom* in all of the columns over the course of the study argues that these organisms can be maintained under environmental conditions for an extended time period (three weeks). There seems to be no other logical interpretation in view of the hybridization data that confirm the presence of *tom* genes as well.

The most logical interpretation of these data would suggest that two processes contributed to the cometabolic degradation of TCE in the soil columns. Because of the high levels of BTEX contaminants in these waters, a significant population of aromatic degraders were probably responsible for the degradation of some of the TCE. How much cannot be determined from these experiments. The added bacteria also clearly survived their introduction and even persisted at very high levels in the most effective treatment column ($PR1_{31}$). Given its continued viability, concentration, and access to nutrients (as demonstrated by its persistently higher oxygen demand), $PR1_{31}$ probably also contributed to the degradation of the TCE seen in this column.

At the very least, this experiment demonstrates a near complete degradation of the water entrained target pollutant TCE (along with DCE, toluene and benzene) under conditions of minimal nutrient amendment, and oxygen levels attainable with an *in situ* groundwater recirculation system during a single 8 hour liquid cycle.

E. CONCLUSIONS

1. TCE, DCE, toluene, and benzene were effectively removed in a soil column model of an in situ groundwater recycle system at NAS Whiting Field

2. Removal rates suggest greater than 95% removal of these contaminants in a single eight hour recycle pass

3. Both indigenous and constitutive bacterial constructs appear to contribute to the cometabolism of the chloroethenes

4. Added nutrients (other than oxygen) did not appear to significantly affect the removal of contaminants

5. The constitutive bacterial constructs appear capable of maintenance and activity for prolonged periods of environmental exposure.

6. It is feasible to apply constructed TCE degrading bacteria to aquifers in a manner suggested by these experiments. The optimal conditions for this application remain to be determined. A continuous or periodic application would be recommended to achieve more significant levels of degraders.

7. Of all strains studied the parent strain PR131 (TOM31c) was maintained at the highest density and maintained the fastest degradation rates over the course of the experiment.

SECTION V.

CONCLUSIONS

1. Direct, one-time application of derivative G4 strains to bioreactors or environmental matrices does not lead to effective degradation of TCE in either liquid or vapor phase.

2. G4 strains do not form an extensive biofilm on any of the support matrices examined, despite its ability to colonize oyster shell to a significant extent under highly buffered conditions.

3. The single greatest weakness seems to be the inability to directly select G4 strains for the constitutive cometabolic activity, which is the only phenotypic marker that differentiates them from the wild type bacteria, and for that reason the only one that matters in these applications.

4. TOM contains the entire Tom operon, and because it is a self transmissible plasmid it is possible to transfer the Tom constitutive expression phenotype to other bacteria by simple bacterial conjugation. This has allowed the creation of other strains expressing this critical Tom constitutive phenotype, that are far better biofilm producers than G4 strains: JS150 (TOM_{31c}) and 249 (TOM_{31c}).

5. The newly created biofilm producing, Tom constitutive strains while capable of forming a highly active biofilm were not competitive with indigenous bacteria under the selective conditions tested.

6. Alginate and polyurethane encapsulation/entrapment methods proved ineffective in retaining $PR1_{23}$ for bioreactor applications. The alginate polymer was too fragile and the polyurethane did not retain the bacteria.

SECTION VI.
RECOMMENDATIONS

Any successful application of the Tom constitutive phenotype (or any other TCE cometabolic phenotype) will first require one of two prerequisites. An engineered environment that protects the host bacterium from environmental pressures of competition and predation and will allow it to survive at functionally significant concentrations (i.e. $>10^8$ cells/ml) (i.e. encapsulation), or a host that can tolerate these negatively selective pressures while degrading TCE. Selective manipulation of alternate bacterial hosts to provide more environmentally stable strains is readily possible because Tom is encoded by the mobile plasmid TOM, which we now know is expressible in other bacteria.

That the enzyme Tom can function effectively under environmental conditions was shown, and clearly points the way to strain selection and improvement.

The results from Whiting field, while somewhat confounded by indigenous TCE degradative capacity, nevertheless indicate that application of the *in situ* recirculation/bioaugmentation process as modeled there, should result in the complete degradation of BTEX and chlorinated ethene contaminants at this site.

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